MUTAGEN SPECIFICITY IN *NEUROSPORA CRASSA*

By

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Submitted to the University of Edinburgh as a Thesis as required for the Degree of Doctor of Philosophy in the Faculty of Science

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1984

This work is dedicated to my mother.

This thesis was composed by myself and describes my own work except where otherwise stated either in the Text or in the Acknowledgements.

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SUMMARY

One approach to understanding the ways in which mutagenic agents cause mutations is to identify the particular base changes which they produce.

The mutagenic specificity of a number of mutagens was investigated using a set of well-characterised mutants of the *am* gene (coding for glutamate dehydrogenase) of *Neurospora crassa*.

On the whole the results show that the mutagens used possess the same specificity in *Neurospora* as they do in other organisms.

The mutagen 4-nitroquinoline-1-oxide (NQO) was used to characterise the nonsense mutant *am 17* as an amber (UAG) rather than an ochre (UAA) mutant. This was on the basis of having demonstrated the specificity of NQO for inducing alterations of G·C base pairs rather than A·T base pairs. NQO was also found to be able to induce frameshift mutations.

Studies with ultra-violet light (UV) show it to be a versatile mutagen able to induce most types of base change. However UV did display a tendency to induce transitions rather than transversions.

Nitrous acid was found to have a strong specificity for inducing A·T → G·C transitions while ethylmethane sulphonate (EMS) appeared to be very specific for the induction of G·C → A·T transitions. EMS was also able to induce frameshift mutations.

The frameshift mutagen ICR-170 was found to be unable to revert the frameshift mutant *am 6*, which has a single base-pair insertion, but was able to revert the mutant *am 15* very efficiently.

The *am 15* mutant was characterised, along with two of its ICR-170 induced revertants, using molecular cloning techniques and the Sanger DNA sequencing method. *am 15*

was found to have a single base-pair deletion and the two revertants were found to have insertions of a G·C base-pair into separate $\frac{-G-G-}{-C-C-}$ sequences.

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ABBREVIATIONS

ATP	adenosine-5'-triphosphate
DEAE	diethyl amino ethyl
DMF	N,N-dimethylformamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
IPTG	isopropyl -β-D-thio-galactopyranoside
kb	kilobase-pairs
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
NBT	nitrobluetetrazolium
^{32}P-dCTP	10μ Ci μl^{-1} α-^{32}P-2-deoxycytosine-5'-triphosphate
PEG	polyethylene glycol
p.f.u.	plaque forming unit
PMS	phenozine methosulphate
SDS	sodium dodecyl sulphate
SSC	0.15M Na Cl, 0.015M sodium citrate
TE	10mM Tris, 1mM EDTA pH 7.5
TEMED	N,N,N',N'-tetramethylethylenediamine
TRIS	tris (hydroxymethyl)-aminomethane
XGAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

MUTAGEN ABBREVIATIONS

2AP	2-aminopurine
5BU	5-bromouracil
EMS	ethylmethane sulphonate
NA	nitrous acid
NQO	4-nitroquinoline-1-oxide
MMS	methylmethane sulphonate
MNNG	nitrosoguanidine
UV	ultraviolet light

CHAPTER I: INTRODUCTION

MUTAGENESIS AND MUTAGEN SPECIFICITY

Mutation can be defined as any heritable variation which arises as a result of alterations in the genetic material of an organism. Most modern definitions of life include mutability as a fundamental property of living organisms.

Mutagenesis has long been studied with respect to its role in the evolution of organisms and more specifically in relation to its role in the study of genetics. The major interest in mutation research today however, arises as a consequence of the important role that somatic mutation appears to play in carcinogenesis. More than 90% of the carcinogens tested have been found to be mutagenic in bacterial systems (McCann *et al.*, 1975). Recent studies have shown that the oncogene responsible for human bladder carcinomas is a normal cellular gene which has undergone an alteration of a single base pair (Tabin *et al.*, 1982). These and other similar findings have led to the idea that studies of mutagenesis could provide us with a better understanding of the nature of carcinogenesis.

One important aspect of mutagenesis is mutagenic specificity. This refers to the tendency of a mutagen to produce one type of mutational damage rather than another. For the purposes of this work mutational damage is restricted to base-pair substitutions and frameshift mutations.

Base-pair substitutions involve the exchange, usually through erroneous pairing during DNA synthesis, of one base pair for another. These substitutions can be classed as transitions, purine-pyrimidine to purine-pyrimidine, or transversions, purine-pyrimidine to pyrimidine-purine.

Frameshift mutations involve the addition or deletion of one or more base-pairs from the coding region of a gene

and usually result in the alteration of the reading frame of the mRNA sequence. In cases where the gain or loss is in multiples of three base-pairs the result is not an alteration in the reading frame, but the addition or deletion of one or more amino-acids from the gene product.

The main aim of this study is to investigate the mutagenic specificity of a number of mutagenic agents in the lower eucaryotic organism Neurospora crassa. This introduction will aim to give the reader an account of the various ways in which mutagenic specificity has been studied in the past, and how recent technological innovations have resulted in far more satisfactory ways of studying the subject. This will be followed by a review of the findings of such studies as they relate to the mutagens used in this work.

But first it would be appropriate to consider the mechanisms by which mutations arise in the genetic material of living organisms as the result of mutagenic attack, and at the same time to look at some of the ways in which the specificity of mutational changes can arise.

MECHANISMS OF MUTAGENESIS

Mutations can be conveniently ascribed to three pathways of mutagenesis; direct mispairing, frameshift mutagenesis, and misrepair.

Direct mispairing

This refers to the misincorporation of bases during DNA synthesis as a result of nucleoside modifications and usually results in specific base-pair substitutions.

Base analogues such as 5-bromouracil and 2-aminopurine give rise to transition mutations through their ability to pair with one particular base in one of their tautomeric states but with a different base in the other tautomeric state.

Most mutagens however give rise to direct mispairing through chemical modifications of bases already present within the DNA helix in such a way as to alter their pairing specificities.

Nitrous acid and hydroxylamine are two well-studied mutagens which modify bases through deamination. Loss of an amino group by cytosine converts it to uracil which pairs with adenine leading to a G·C → A·T transition. Adenine can be similarly converted to hypoxanthine which pairs with cytosine leading to an A·T → G·C transition. Deamination of the other base which possesses an amino group, guanine, does not have mutagenic consequences since the pairing specificity of the base is not altered.

The largest class of mutagens are the alkylating agents which can attach alkyl groups to many positions on the DNA molecule including all the nitrogen atoms, the O-6 position of guanine, and the O-2 and O-4 positions of thymine (Singer, 1977). The major product of alkylation is N7-alkylguanine but this has been shown not to promote mis-incorporation (Ludlum, 1970). Only two of the many alkylation products are likely to cause direct mispairing and these are O6-alkylguanine and O4-alkylthymine (Lawley, 1974; Drake & Baltz, 1976). O6-alkylguanine is able to pair with thymine and thus gives rise to G·C → A·T transitions, while O4-alkylthymine is able to pair with guanine which leads to A·T → G·C transitions (Lawley et al., 1973) O4-alkylthymine is found to be present in relatively low amounts of double-stranded DNA, but is found at considerable levels in single-stranded DNA such as in replicating regions (Singer, 1977). O6-alkylguanine is probably the more important mispairing lesion since there is a predominance of G·C → A·T transitions in mutagenesis by alkylating agents (Coulondre & Miller, 1977). There also appears to be a strong correlation between the proportion of alkylation at the O6 position of guanine and the mutagenicity of the alkylating agent (Singer, 1977).

O6-alkylguanine can be removed from E. coli DNA by an inducible repair process which is expressed after exposure of cells to low doses of alkylating agents (Cairns, 1980). This process, called adaptive response, is error-free and involves the induction of a 'suicidal' DNA methyltransferase which accepts the alkyl group from O6-alkylguanine to a cysteine residue in the enzyme thereby inactivating it (Lindahl, 1982).

Many chemically modified bases can be removed directly from the DNA of E. coli through the action of DNA glycosylases which catalyze the cleavage of base-sugar bonds to generate apurinic or apyrimidinic sites. Many DNA glycosylases have been discovered and each appears to be specific for the removal of particular lesions. For example, uracil-DNA glycosylase and hypoxanthine-DNA glycosylase can remove deaminated cytosine and adenine respectively (Duncan et al., 1978; Karran & Lindahl, 1978). Both enzymes have been isolated from mammalian cells as well as from E. coli (Lindahl, 1982). Other glycosylases exist which can remove alkylating damage to DNA. However these tend to be specific for those lesions which are potentially lethal, such as 3-methyl-adenine and 7-methylguanine, rather than those which give rise to direct mispairing.

In Neurospora mutants have been isolated which are sensitive to the mutagenic effect of MMS (Kafer, 1978), MNNG (Inoue et al., 1975), nitrogen mustard (Schroeder, 1970), all of which are alkylating agents, and NA (Tuveson, 1972). Since these mutants are all concerned to a certain extent with excision of damage it seems safe to assume that Neurospora also possesses several repair processes by which mispairing lesions can be removed.

Frameshift mutagenesis

While working with the bacteriophage T4, Streisinger discovered that frameshift mutations had a tendency to occur in tandemly repeated base sequences (Streisinger, 1966). This led him to suggest that frameshifts occur when strand breakage in the vicinity of such repeated sequences allows base-slippage and the looping out of one or more bases. Repair of the breaks would then lead, after a round of replication, to an addition mutation if the loop-out is in the same strand as the break, or a deletion mutation if the loop-out is in the opposite strand.

Further studies have shown that repeated base-sequences are hot-spots for frameshift mutagenesis in the plasmid pBR322 (Fuchs *et al.*, 1981), *Salmonella* (Young & Kallenbach, 1981; Isono & Young, 1974) and *E. coli* (Calos & Miller, 1981) thus supporting the Streisinger model.

The most potent frameshift mutagens in prokaryotes are a group of chemicals known as acridines. These are especially potent when they have an alkylating group attached as in the ICR acridine compounds. It was first suggested that these compounds induced frameshifts by intercalating into regions of base slippage and stabilising them until the mutations were fixed (Lee & Tinoco, 1978). In some models it was suggested that particularly effective mutagens such as 9-aminoacridine could further stabilise a looped out guanine residue by binding to the unpaired cytosine by virtue of an amino group (Young & Kallenbach, 1981). However not all intercalating chemicals are frameshift mutagens. This has led to the further proposal by Streisinger that frameshift mutagens stabilise base-slippage intermediates not by intercalating but rather by extrahelical stacking with the looped out bases (Drake & Baltz, 1976).

In *E. coli* mutants which are defective in the excision repair process show increased mutation with ICR-191 whereas mutants of the error-prone repair pathway, controlled by

recA, show no change in their response to this mutagen (Newton et al., 1972). This suggests that in bacteria acridine lesions can be removed by excision enzymes but that if the lesions survive into replication frameshift mutation occurs independently of error-prone repair.

In Neurospora however, no mutants have been isolated which show increased mutation with ICR-170 but the mutant uvs3, which is similar in some ways to recA in E. coli, is immutable with ICR-170 (Inoue et al., 1981). This suggests that ICR frameshift mutagenesis in Neurospora requires some cellular function which is absent in uvs3. In yeast frameshift induction by ICR-170 is reduced but not abolished in a rad6 mutant. The RAD-6 gene is thought to be involved in error-prone repair processes (Lawrence, 1982) which suggests that a proportion of ICR mutagenesis in yeast occurs through the participation of an error-prone repair process.

Mutagens such as UV, nitrous acid and MMS can also induce frameshift mutations in bacteria (Le Clerc & Istock, 1982; Miller, 1983), Neurospora (Siddig et al., 1980) and yeast (Sherman & Stewart, 1973). Some of these fall within repetitive base sequences and are therefore consistent with the Streisinger model, as long as it is assumed that repair of the lesions produced by these agents can create the conditions under which base-slippage occurs. Some of the mutations however are not consistent with the Streisinger model in that they do not occur within repeated base sequences. It has been suggested that apurinic or apyrimidinic sites created through excision of various types of lesion by glycosylases, or through spontaneous loss of unstable chemically modified bases may be a source of base deletion mutations. Apurinic sites have been shown to be mutagenic in E. coli cells which have had the 'SOS' error-prone repair pathway induced (Shaaper et al., 1982).

More complex frameshift mutations involving concomitant insertion and deletion of bases and maybe also base substitutions sometimes occur and are difficult to explain. Such mutations have been observed in Neurospora (Siddig et al., 1980) and yeast (Sherman & Stewart, 1973). Ripley (1982) has proposed that such mutations may come about through the formation of secondary structures such as hair-pin loops from quasi-palindromic sequences. It is suggested that imperfections in these secondary structures such as looped out bases and mismatched bases could be removed by repair enzymes to generate both frameshifts and base-substitutions. A complex yeast mutation involving the substitution of an ACCT sequence for a CGG sequence is explicable in terms of this model.

Misrepair

Mutations arising through misrepair are generally thought to be the byproducts of error-prone repair systems which sacrifice the integrity of DNA sequences in order to circumvent potentially lethal mutagenic lesions.

In E. coli an inducible error-prone repair system was first observed when UV mutagenesis was found to be controlled by the genes recA and lexA (Witkin, 1976). Radman called this the 'SOS' repair system. It is now known that the recA and lexA genes control a complex regulatory system involving a large number of genes encoding a wide variety of cellular responses (Little & Mount, 1982). This includes the genes uvrA and uvrB which code for the endonuclease complex required for the removal, by excision repair, of bulky lesions, such as those caused by UV, NQO and mitomycin C, from the DNA (Sancar et al., 1982). The error-prone component of SOS repair has been found to be dependent on the locus umuC (Bagg et al., 1981). It appears that the umuC locus comprises two genes since two proteins have been isolated as products of the locus (Bridges & Lehmann, 1982). These products together with the recA gene product appear to play a key mechanistic

role in error-prone repair. It has long been thought that the error-prone nature of SOS repair is due to the relaxation of fidelity of DNA replication which enables synthesis past a lesion which is blocking the replication machinery, at the possible expense of incorporating a mistake. This theory has been supported by the recent observation and isolation of a variant of DNA polymerase I which possesses lower fidelity of replication and is associated with the induction of SOS functions (Lackey et al., 1982).

The type of lesions which are bulky enough to block replication include those produced by UV, NQO, mitomycin C and ICR compounds, as well as a large range of other mutagenic and carcinogenic compounds. Some alkylations are found to be able to distort the structure of the DNA sufficiently to interfere with normal replication, and apurinic gaps, which can result from alkylation, are able to induce SOS repair (Shaaper & Loeb, 1981).

It would appear that those lesions which are able to induce SOS repair by interfering with replication can be removed prior to replication by excision repair. This consists of an incision in the vicinity of the lesion by an endonuclease, which may be specific for the lesion being removed (Warner et al., 1980), followed by removal of the damage by an exonuclease, resynthesis of the excised region by a DNA polymerase, and finally joining of the new section to the old by a ligase. Recent studies have shown that the products of the genes uvr A, B and C, which are required for the excision repair of most bulky lesions, form an enzyme complex that is able to nick the DNA on both sides of a UV-induced pyrimidine dimer and release a twelve or thirteen base oligonucleotide containing the damage (Sancar & Rupp, 1983). Resynthesis of the excised region is probably carried out by DNA polymerase I, mutants of which show decreased excision capability (Cooper & Hanawalt, 1972a). On the

whole excision repair pathways are error proof, but a low level of mutation has been ascribed to the repair of a low proportion of excision gaps (Bridges & Mottershead, 1971). This error-prone excision repair appears to involve repair patches of several hundred nucleotides as opposed to the usual ten to thirty nucleotides and is hence known as long patch excision repair (Cooper & Hanawalt, 1972b). It also requires the wild type function of the SOS repair genes *recA* and *lexA*. Sedgewick (1976) has suggested that the substrate for error-prone SOS repair in bacterial DNA is closely spaced blocking lesions on opposite strands of the DNA. Thus during excision repair if the normal removal of one lesion produces a single-stranded gap opposite a second lesion, then normal gap-filling can not proceed past this second lesion. This single-stranded gap would be lethal if allowed to enter the replication cycle and so it is likely that some SOS function is called upon to fill in the gap at the possible expense of incorporating a mistake.

Closely spaced blocking lesions on opposite strands of the DNA are also thought to be responsible for SOS mutagenesis during replication. When a solitary blocking lesion enters replication a gap will be left in the newly synthesised daughter strand opposite the lesion. Such gaps were found to gradually disappear during the hour following DNA replication (Rupp & Howard-Flanders, 1968) and are the substrate for a recombination process which is dependent on the DNA recombination properties of the *recA* gene product (Radding, 1981). Strand exchange takes place between the gapped daughter strand and the parental strand of like polarity (West *et al*., 1981). Thus the gap is transferred to the sister duplex where it can be filled in by DNA polymerase using the intact and lesion-free daughter strand. The lesion is thus circumvented and is accessible to excision repair processes before the next round of replication.

If, however, two closely spaced blocking lesions on opposite strands are allowed to enter the replication cycle they will result in overlapping daughter strand gaps which will be refractory to the recombination dependent gap transfer outlined above. Thus error prone SOS repair would be required to restore the gapped daughter duplexes to the double stranded condition, despite the increased risk of incorporating errors.

The hypothesis that closely spaced lesions are the substrate for error prone SOS repair is supported by the finding that UV light induces mutations in E. coli with two-hit kinetics (Witkin, 1969). However, the tif mutant, in which SOS functions are thermally inducible, displays linear kinetics of UV mutagenesis suggesting that SOS error prone repair can compete successfully with error free repair processes once it has been induced (Witkin, 1974).

Misrepair mutagenesis like that which occurs in E. coli has been observed in a wide variety of organisms (Drake & Baltz, 1976). In Neurospora the mutant uvs3 behaves like the recA mutant of E. coli in that it is completely resistant to UV-induced mutation but is sensitive to killing by UV (De Serres, 1980). Both mutants also show this same response with NQO (Inoue et al., 1980).

Two Neurospora mutants, uvs-2 and upr1, have been isolated which are unable to excise dimers (Worthy & Epler, 1973) and are sensitive to UV and NQO inactivation and mutation induction (Inoue et al., 1980). Thus there are obvious similarities in the ways E. coli and Neurospora process mutational lesions. However there are also important differences. The uvs3 mutant is completely resistant to ICR and MNNG mutagenesis (Inoue et al., 1980) which suggests that the uvs3 gene product is required for frameshift mutagenesis and some types of

alkylation mutagenesis. This latter suggestion would mean that direct mispairing is significantly reduced in Neurospora.

In yeast the rad6 mutant is completely resistant to UV mutagenesis and very sensitive to UV inactivation and thus appears to control an error-prone repair pathway (Lawrence et al., 1974). There appears to be almost a total requirement for the rad6$^+$ function for mutagenesis by any mutagenic agent. The most notable exception is ICR-170 which is able to induce frameshift mutations in a rad6$^-$ strain (Lawrence, 1982). This not only suggests that direct mispairing as a source of mutations is absent in yeast, but also that ICR-170 produces frameshifts in a different manner to mutagens such as UV and MMS. A large number of mutants have been isolated in yeast which are concerned with excision repair and which appear to excise damage induced by most of the well-used mutagens (Cox & Game, 1974).

In E. coli there is an additional excision repair process called post-replicational mismatch repair which acts upon incorrectly inserted or mismatched bases in newly synthesised DNA. Details of the process are poorly understood at present, but judging from the number of genes involved, known as mut genes, it is a fairly complex system. It appears that the mismatch excision machinery is able to recognise mismatched base-pairs and also to distinguish the daughter strand from the parental strand and hence identify the incorrectly inserted base. This discrimination appears to be the result of a delay in post-replicational methylation of the daughter strand. Methylation occurs at the N6 position of adenine in the sequence GATC and is carried out by the enzyme DNA adenine methylase. Mutants which are deficient in this enzyme, dam$^-$ mutants, show increased spontaneous mutation frequencies, increased mutation frequencies with the base analogues 2AP and 5BU, and increased mutation frequency

with the alkylating agent EMS (Glickman, 1982).

Direct proof of a mismatch repair system in lower eucaryotes is unavailable at the present time. Methylation of eucaryotic DNA does take place and various functions have been ascribed to this, including the control of gene expression. Mismatch repair seems to be such a useful process that it is not hard to suppose that eucaryotes might possess a similar system, if not a similar mechanism, of strand discrimination.

From this brief review of the ways in which various mutagenic lesions lead to mutations, and the ways in which organisms attempt to remove these lesions before they become fixed as mutations, it must be clear that the spectrum of mutations induced by a given mutagen is dependent on the operation of various complex cellular responses to mutagenic attack and also the primary reaction between the mutagen and the genetic material. Thus the analysis of the final spectrum of mutations induced by a mutagen can provide us with important clues as to the nature of the mutational process itself, and to the nature of the repair processes employed by organisms to protect the integrity of their genetic material.

For instance studies have shown that in the lacI gene of E. coli EMS induces almost exclusively G-C → A-T transitions (Coulondre & Miller, 1977). Biochemical evidence tells us that EMS can alkylate the O6 position of guanine and that this alkylated guanine can directly mispair with thymine (Singer, 1977). Thus it is concluded that the major mutagenic product of EMS is O6 alkylguanine and if this is not removed by the cell a G·C → A·T transition may result. However recent studies have shown that in a dam mutant of E. coli, which is deficient in mismatch repair, 15% of the mutations induced by EMS are transversions (Glickman, 1982). This

suggests that EMS may produce lesions which lead to transversions in E. coli but that these lesions are very efficiently repaired by the mismatch repair mechanism.

Another example of how the analysis of mutations can provide clues to the nature of the mutational process comes from work that was carried out on the mutational specificity of UV light in E. coli (Todd and Glickman, 1982). It was discovered that the four most UV-mutable sites within their system fell within quasi-palindromic sequences which could, potentially, form looped-out secondary structures. This finding prompted the suggestion of a role for secondary structures in determining hot-spots for UV mutagenesis within DNA.

METHODS FOR STUDYING MUTAGEN SPECIFICITY

It is now appropriate to examine the ways in which, over the years, workers have attempted to investigate the problem of mutagen specificity. As will be seen the current advances in DNA technology have facilitated the development of powerful systems of investigation.

In the early days of mutagen specificity studies it was not possible to define precisely the base changes induced or reverted by mutagens. Instead workers used a set of standard mutagens whose biochemistry was so well understood that it was felt that the base-changes produced by these mutagens could be confidently predicted. These standard mutagens were then used to characterise other less well understood mutagens by carrying out comparative studies. For example the base analogues 2AP and 5BU were thought to possess base-pairing ambiguities and as a result would lead to transition mutations, i.e. A·T → G·C and G·C → A·T. Another mutagen, hydroxylamine, was thought to deaminate cytosine to uracil, which pairs with adenine, leading to G·C → A·T transitions.

This type of comparative analysis has been carried out, using extensive sets of standard mutagens in some cases, in a wide range of organisms including bacteriophage (Drake, 1963; Bautz & Freese, 1960; Tessman *et al*., 1964; Ishizawa & Endo, 1971), bacteria (Eisenstark *et al*., 1965; Oeschger & Hartman, 1970) and *Neurospora* (Malling & De Serres, 1968; Brusick, 1969; Kilbey *et al*., 1971). In *Neurospora* and yeast complementation studies of mutants also yielded information as to their likely nature (De Serres *et al*., 1971; Ong *et al*., 1975). For instance base substitutions could complement other mutants while frameshifts were less likely to.

While such studies provided a lot of useful information as to the probable molecular nature of various induced mutations, too many ambiguities persisted. Thus workers have turned to systems in which the precise molecular nature of mutations could be established.

Osborn *et al*. (1967) used a system in which the nonsense suppressors induced in *E. coli* by a mutagen could be characterised using amber and ochre mutants of T4 bacteriophage, with the base changes involved inferred by identifying the amino acids inserted opposite the nonsense codons. One interesting result which arose from these studies was that the base analogues 5BU and 2AP, thought to be able to produce both types of transition equally, induced A·T → G·C transitions 83% and 95% of the time respectively. Another result indicated that hydroxylamine, used as a standard mutagen for inducing G·C → A·T transitions, was able to induce twice as many A·T → G·C transitions as G·C → A·T transitions. These results illustrate the point that mutagens do not always behave *in vivo* as they are expected to on the basis of their *in vitro* biochemistry.

Ishizawa and Endo (1970) used a set of ten T4 bacteriophage mutants for which the base changes required for reversion to wild-type were known. This set of

mutants was then used to determine the specificity of mutagens on the basis of their ability to revert the different mutants back to wild-type.

Coulondre and Miller (1977) used an extensive set of over 40 amber and ochre mutants of the lacI gene of E. coli to study mutagen specificity. The base change required to generate each of the nonsense mutants was known. Mutagen specificity was then studied by determining which nonsense mutants, and hence which base changes, could be induced by a particular mutagen.

The first study of this sort to be performed in a eucaryote was by Sherman and his colleagues using well-characterised mutants of the iso-1-cytochrome C gene of yeast. The base changes required to revert these mutants to wild-type were deduced from amino-acid sequencing of the altered peptides from non-wild-type revertants (Sherman & Stewart, 1973). This set of mutants, eleven in all, were then used to screen a large number of mutagens whose specificity was determined from their ability to revert each of the mutants.

More recently, with the advent of techniques that allow fast and efficient sequencing of DNA, it has become possible to sequence forward mutations to auxotrophy without the need for constructing sets of well-characterised mutants to study specificity.

Several groups have taken advantage of this new technology. Fuchs et al. (1981) have carried out an extensive study of the specificity of the carcinogen N-acetoxy-N-2-acetylaminofluorene in the bacterial plasmid pBR322 by analysing forward mutations induced in the tetracycline resistance gene of the plasmid.

Le Clerc and Istock (1982) and also Demopoulos (1982) have both developed rapid sequencing systems for studying mutagen specificity in the sequencing vector bacteriophage M13. Forward mutations are easily isolated and the single-

stranded DNA phage is easily sequenced.

Sherman (see above)has now developed a fast cloning and sequencing system for isolating and characterising mutants arising in the iso-l-cytochrome C gene of yeast (personal communication).

While it is unnecessary to review the specificities of all the mutagens investigated in these studies, it is nevertheless of interest to look at how such studies have contributed to our current understanding of the specificity of the five common mutagens used in this study: UV, EMS, NQO, ICR-170 and NA.

MUTAGEN SPECIFICITY OF FIVE COMMON MUTAGENS

Ultraviolet light

UV light has often been considered to be an all-purpose mutagen, capable of inducing most types of mutation ranging from base-pair substitutions and frame-shifts to multi-locus deletions and translocations. While it is evident that UV is capable of inducing a wide spectrum of mutations (Le Clerc & Istock, 1982; Glickman, 1983; Sherman & Stewart, 1973), it is also apparent that at least in prokaryotic systems, UV shows a pronounced specificity for transition mutations.

In the cI gene of λ bacteriophage two-thirds of UV-induced mutations are transitions (Hutchinson, F., Personal communication), while in the lacI system of E. coli most mutations involved G·C → A·T transitions (Miller, 1983; Todd & Glickman, 1982). Earlier studies also indicated a preference for G·C → A·T transitions (Howard & Tessman, 1964).

For a long time it was thought that pyrimidine dimers were the sole lesions responsible for UV muta-genesis. Photoreactivation, which removes dimers from the DNA, reverses the mutagenic effects of UV. It is

the inhibitory effect by dimers on DNA synthesis which is thought to induce the SOS error-prone repair system (Witkin, 1976). This in turn is thought to involve a reduction in DNA polymerase fidelity which facilitates synthesis past blocking lesions. It is this reduction in fidelity that is held to be responsible for the high incidence of 'untargeted' UV mutations, i.e. mutations occurring at sites in the DNA where dimers are unable to form (Brandenburger, 1981; Schaaper & Glickman, 1982).

It is difficult to explain how error-prone synthesis past UV dimers could lead to the observed specificity of UV mutagenesis. In the past few years, however, attention has focussed on another UV induced lesion which may be able to account for this specificity. This is the so-called P_Y-C* photoproduct which occurs at pyrimidine-cytosine sequences (Brash & Haseltine, 1982). In the cI gene of λ bacteriophage a treatment which induces mostly dimers produced mainly transversion. However, phage which were photoreactivated to leave only P_Y-C* products underwent mostly transitions (Hutchinson, F., Personal communication). In another study using the lacI gene of *E. coli* hot-spots for dimer formation did not correlate with mutation hot-spots, whereas hot-spots for P_Y-C* products did (Brash & Haseltine, 1982). Also in the lacI gene 90% of G·C → A·T transitions were found to occur at potential P_Y-C* sites (Todd & Glickman, 1982).

It seems possible, therefore, that in prokaryotes UV induces mainly transitions, and that these transitions are mostly targeted at sites where P_Y-C* photoproducts are able to form. This hypothesis would be reinforced if it could be shown that the modified cytosine in the P_Y-C* sequence can mispair with adenine and hence give rise to a G·C → A·T transition.

In the light of the evidence on P_Y-C* photoproducts the role of dimers becomes less clear. Dimers are able to induce the SOS repair functions and these, in a tif

mutant where SOS functions are thermally inducible, show a specificity for G·C → T·A transversions (Miller, 1983). It may be the case that dimers contribute mostly to the lethal effects of UV light, such as strand breaks, and that Py-C* photoproducts and error-prone SOS functions account for most of the observed transitions and transversions respectively.

The most extensive study of UV frameshift specificity in bacteria has been carried out in the lacI gene of E. coli (Miller, 1983). This study indicates that UV light induces frameshifts mostly at monotonous runs of GC and AT base pairs. This is in accordance with Streisinger's model of frameshifting through base-slippage intermediates (1966). However, there was a bias towards mutations at runs of AT base-pairs, and a ten to twenty-fold bias towards single base-pair deletions. The reasons for this bias are unclear.

Although a lot of work has been done on the repair of UV damage in eucaryotes little is known about UV specificity. The best study to date was conducted on the iso-1-cytochrome C system of Sherman and Stewart (1973). In this study UV was able to revert mutants requiring all four types of transversion and both types of transition to revert to wild-type. UV was also able to induce frameshifts at a thymine-thymine sequence but also at sequences where base-slippage could not occur. UV was also able to induce more complex frameshifts.

In the am gene of Neurospora UV was able to delete single bases from repetitive sequences, and also, as in yeast, to induce more complex frameshifts (Siddig et al., 1980).

Ethylmethane sulphonate

EMS is an alkylating agent which is able to alkylate at many positions within a DNA molecule.

In the lacI system of Coulondre and Miller (1977)

EMS is highly specific for the induction of G·C → A·T transitions with the only other induced changes being A·T → G·C transitions which made up less than 1% of the total.

In the iso-1-cytochrome C system of yeast EMS was only able to revert those mutants which reverted via a G·C → A·T transition (Prakash & Sherman, 1973).

This specificity is probably due to one of the alkylation products of EMS, namely O6-ethylguanine (Singer, 1977). This modified guanine is able to mispair with thymine giving rise to G·C → A·T transitions (Lawley *et al.*, 1973). The small percentage of A·T → G·C transitions seen with EMS could be due to the mispairing properties of another of its alkylation products, O4 ethyl thymine.

4-Nitroquinoline-1-Oxide

NQO is of special interest because its mutagenic pathway is very similar to that of UV light; it produces excisable lesions and induces the SOS response (Ikenaga *et al.*, 1975; Inoue *et al.*, 1980).

The active mutagen appears to be a metabolic derivative of NQO which covalently binds to guanine and, to a lesser extent, adenine (Ikenaga *et al.*, 1975).

In the *lacI* system of Coulondre and Miller the vast majority of NQO mutations are G·C → A·T transitions, with the rest made up of mostly GC transversions and a very small proportion of AT transversions (Miller, 1983). It must be remembered however that this system does not screen for A·T → G·C transitions.

Also in the *lacI* gene of *E. coli*, out of 223 NQO induced mutants, 76% were G·C → A·T transitions, with only 3% being A·T → G·C transitions. The other 21% were ambiguous being either G·C → T·A or A·T → T·A transversions (Shinoura *et al.*, 1983).

In bacteriophage T4 NQO was able to revert four

mutants with GC at the mutant site, but was unable to revert four mutants with AT at the mutant site and two frameshifts (Ishizawa & Endo, 1970).

In the iso-1-cytochrome C system of yeast NQO was able to revert only those mutants requiring GC transitions or transversions to return to wild-type (Prakash et al., 1974).

The clear specificity of NQO is apparently explained in terms of its preferential attack on guanine residues and the incorporation of mispaired bases opposite the modified guanine by the SOS repair functions. The observation that thymine is preferentially incorporated opposite NQO-guanine lesions may mean that these lesions are not totally non-instructive.

NQO exhibits some frameshift activity in some systems. In Salmonella NQO was able to delete the sequence CG from the repetitive sequence CGCGCGCG (Isono & Yourno, 1974).

ICR-170

ICR-170 is a member of a group of carcinogens which are made up of alkylating side chains attached to acridine rings.

Acridine compounds are known to be potent frameshift mutagens (Ames & Whitfield, 1966). Streisinger has suggested that they induce frameshifts by stacking extra-helically with looped-out bases in the vicinity of DNA strand breaks and hence stabilise them while the looped out structure is fixed as a mutation (Drake & Baltz, 1976). This looping-out or base-slippage is thought to occur preferentially at repetitive base sequences.

ICR compounds specifically induce frameshifts in repetitive runs of GC base-pairs. In Salmonella, ICR-191 and ICR-364-OH are able to insert a cytosine into a run of three cytosines, and are also able to delete a CG from a CGCGCGCG sequence (Isono & Yourno, 1974).

In the *lacI* gene of *E. coli* 98% of ICR-191 mutations are single base deletions or additions from either GGG or GGGG sequences (Calos & Miller, 1981).

The reason for the specificity of ICR compounds for runs of G·C base-pairs is thought to be that the alkylating side chains of these compounds preferentially attack guanine residues and thus are able to target the acridine rings onto runs of G·C base-pairs. Why this should be the case when alkylating agents are also known to attack adenine residues is not clear.

In *Neurospora* ICR-170 appears predominantly to induce mutations of the frameshift type (Brusick, 1969) although no sequence data are available for this organism.

In yeast Stewart and Sherman (1974) found that ICR-170 was unable to induce mutations in a region of the iso-1-cytochrome C gene containing the sequence GGCCGG. This is surprising since other mutagens were able to induce frameshifts in this sequence, and evidence from prokaryotic studies would suggest that this sequence should be a prime site for ICR mutagenesis.

Nitrous Acid

Nitrous acid is a compound which is able to react with primary amines by deamination to replace an amino group with a hydroxyl group.

Thus the principal mutagenic reaction of nitrous acid with DNA is thought to be with the bases adenine and cytosine. Deamination of adenine converts it to hypoxanthine which pairs with cytosine so leading to an A·T → G·C transition. Deamination of cytosine converts it to uracil which pairs with adenine and leads to a G·C → A·T transition. Thus nitrous acid should theoretically be able to induce both types of transition.

In yeast nitrous acid is able to induce A·T → G.C transitions (Stewart et al., 1972) and G·C → A·T transitions (Prakash & Sherman, 1973). However, nitrous acid

was also able to induce the mutant cycl-6 which arose via a G·C → C·G transversion.

In studies carried out in Neurospora nitrous acid was unable to revert any of twenty-four mutants isolated after ICR-170 treatment (Brusick, 1969) which suggests it is not a very good frameshift mutagen. However, in yeast nitrous acid was able to revert frameshifts by inserting or deleting bases in GC and AT repetitive sequences (Stewart & Sherman, 1974).

The Neurospora am System

Two things should be fairly clear from this brief review of common mutagen specificities; firstly that the conclusions drawn about mutagen specificities are based on comparatively few studies within even fewer systems, and secondly that very few results have been obtained with eucaryotic systems.

The only eucaryotic system to have yielded information on mutagen specificity using well-characterised mutants has been the iso-1-cytochrome C system of Sherman and colleagues (Sherman & Stewart, 1973). The ad3A & B system of De Serres (1971) has provided a number of clues about mutagen specificity in Neurospora but no specific base sequence data.

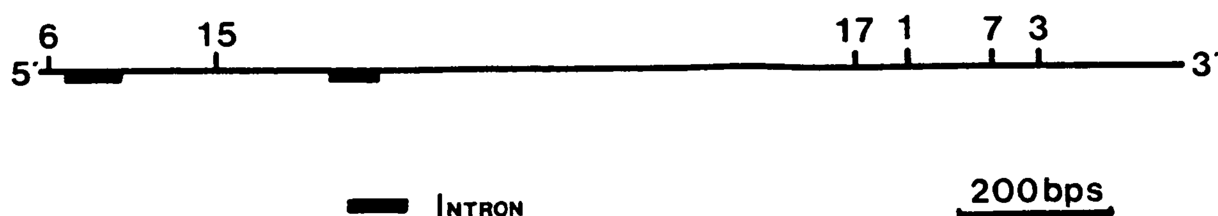
This piece of work therefore has a very straightforward aim, and that is simply to provide more information on the specificity of mutagenesis in eucaryotic organisms by using a system developed in the am gene of the lower eucaryotic fungus Neurospora crassa.

The am gene of Neurospora crassa codes for the enzyme NADP-specific glutamate dehydrogenase and is one of the best characterised of eucaryotic genes. The gene has been cloned (Kinnaird et al., 1982) and completely sequenced (Kinnaird & Fincham, 1983). A number of mutants have been mapped within the gene and their base alterations inferred from amino-acid sequencing studies of the

mutant enzymes (Brett *et al*., 1976). Some of these mutants make up the system used for studying mutagen specificity in this project and are presented in Figure 1.

The basic procedure used to investigate mutagen specificities using well characterised mutants is to carry out reversion analyses to establish which base changes can be induced by which mutagens. In a number of cases where reversion to wild-type phenotype can be achieved through secondary suppressor mutations it is necessary to carry out enzyme assays to distinguish true wild-types from pseudo-wild-types.

In the study on frameshift mutagenesis the approach taken has been to clone and sequence the possible frame-shift mutant *am15* and its revertants in order to discover the nature of the sequence changes involved.



am1 SER 336 (TCC) → PHE (TTC) Brett et al 1976

am3 GLU 393 (GAG) → GLY (GGG) .. .. ..

am7 GLY 372 (GGT) → SER (AGT) .. .. ..

am17 GLN 313 (CA$^{A}_{G}$) → TERM. (TA$^{A}_{G}$) Seale et al 1977

am6 Insertion of C into SER 5 (TCT) Siddig et al 1980

am15 ?

FIGURE 1. Map of well-characterised am mutants used in tester system and sequence alterations associated with each mutant.

CHAPTER II: BASE-SUBSTITUTION MUTAGENESIS - THE CHARACTERISATION OF A NONSENSE MUTANT

(i) INTRODUCTION

Occasionally a strain can undergo a mutation which converts an amino-acid codon into a termination codon, usually with disastrous results for the gene product. This type of mutant is called a nonsense mutant. There are three possible termination codons; amber-UAG, ochre-UAA, and opal-UGA. The two commonest ways in which such mutants can revert are via a mutation within the nonsense codon converting it back into an amino acid codon, or via a mutation at another locus which suppresses the expression of the nonsense mutation. This latter situation has been shown to be the result of a mutation in the anticodon of a tRNA gene which allows the tRNA molecule to read the nonsense codon as an amino acid codon (Piper et al., 1976).

One of the mutants used in this system, am 17, is a nonsense mutant. Seale (1968) first demonstrated this by showing that am 17 could have its GDH activity restored through a mutation at an unlinked locus. This suppressor mutation, when isolated, was also able to suppress mutants in the aro gene cluster in Neurospora (Case and Giles, 1968). Subsequently, seven different 'supersuppressors' have been identified; ssu-1 to ssu-7. These are able to suppress nonsense mutations in a range of loci including am, aro, his-3, trp-1 and trp-2.

It is not known whether these Neurospora ssu loci suppress amber, ochre or opal mutations. In order to determine this, it would be necessary to discover the sequence of the nonsense codons in the suppressible mutants.

Seale et al. (1977) came close to finding the sequence of the am 17 nonsense codon when it was shown that in two classes of am 17 revertants the nonsense codon had been replaced by tyrosine and leucine codons. This showed that the nonsense codon was most likely to

be either amber (UAG) or ochre (UAA) since these are able to give rise to both leucine (UU^{A}_{G}) and tyrosine (UA^{U}_{C}) through single base-pair changes. Opal (UGA) can only give rise to a tyrosine codon via a double base-pair change which is considered to be a rare mutational event.

If Seale had managed to find a revertant class with a tryptophan (UGG) replacement then this would have pointed strongly to the nonsense codon being amber (UAG) and not ochre (UAA). However, out of twenty-two revertants which were analysed none turned out to have a tryptophan replacement. This could mean one of three things; firstly, that the nonsense codon is ochre and is unable to mutate to the tryptophan codon via a single base-pair change; secondly, that the codon is amber but that a tryptophan replacement leads to an inactive enzyme; or thirdly, that the codon is amber but the sample was not big enough to pick up tryptophan revertants.

An alternative solution to the problem of distinguishing amber and ochre codons from one another involves an interesting exercise in mutagen specificity. A mutagen which was specific for G-C base-pairs might be expected to revert an amber nonsense codon, containing a G-C base-pair, but not an ochre codon, lacking a G-C base-pair. However a G-C → A-T transition of an amber codon would simply change it into an ochre codon. Thus a mutagen capable of inducing a G-C → T-A or C-G transversion would be required to distinguish amber and ochre codons on the basis of their ability to revert to a wild-type phenotype.

The mutagen which fulfils these requirements most closely is 4-nitroquinoline-1-oxide (NQO). From studies with mutations of T4 bacteriophage, Ishiyawa and Endo (1970, 1971) concluded that NQO acts preferentially on G-C base-pairs to give rise to G-C → A-T transitions. Prakesh _et al_. (1974) also demonstrated NQO's specificity

for G-C base-pairs, but showed that it could also induce transversions of G-C base-pairs, through its ability to revert six amber mutants but none of nine ochre mutants in their yeast iso-1-cytochrome-C system. One clue to understanding the reasons for the apparent specificity of NQO comes from work done by Ikenaga et al. (1975). They showed that the main lesions induced in DNA by the treatment of E. coli by NQO are an NQO-guanine adduct, and an NQO-adenine adduct, and that these lesions occur in the ratio of about 7:1. Thus NQO appears to covalently bind preferentially to guanine in E. coli, and perhaps induces the replication machinery to insert the wrong bases opposite the affected guanine residues.

If NQO has the same specificity in Neurospora as it does in other organisms, then it would be expected to revert the mutant am 17 with good efficiency if it is an amber mutant but not very well if it is an ochre mutant. A further expectation if am 17 were an amber mutant would be that most of the NQO-induced revertants should have tyrosine replacements since tyrosine codons are generated by GC transversions of amber codons (see Fig. 2.1a). On the other hand there would be no expected specificity of amino-acid replacement if NQO were able to revert an ochre codon since all three positions of the codon should be mutable at a similar, possibly very low, frequency (see Fig. 2.1b).

Thus if NQO was found to revert am 17 it would be important to characterise the amino-acid replacements of the revertants in order to identify the base-changes involved.

The work of Seale et al. (1977) has made available three am 17 revertant strains with known amino-acid replacements; RN35, which has a tyrosine replacement, RU9, which has a leucine replacement, and am 17 ssu 1 which has a supersuppressor which allows for the insertion of tyrosine opposite the nonsense codon. The tyrosine and

FIGURE 2.1 Base Changes Involved in Reversion of am 17 Amber and am 17 Ochre Codons

(a) Am 17 Amber:

CAG → UAG →	UA^{U}_{C}	(TYR)	GC		TV
(GLN) (AMB)	UUG	(LEU)	AT →	TA	TV
	CAG	(GLN)	AT →	GC	TI
	AAG	(LYS)	AT →	TA	TV
	GAG	(GLU)	AT →	CG	TV
	UCG	(SER)	AT →	CG	TV
	UGG	(TRP)	AT →	GC	TI
	UAA	(OCHRE)			

(b) Am 17 Ochre:

CAA → UAA →	UA^{U}_{C}	(TYR)	AT		TV
(GLN) (OCH) →	UUA	(LEU)	AT →	TA	TV
→	CAA	(GLN)	AT →	GC	TI
→	AAA	(LYS)	AT →	TA	TV
→	GAA	(GLU)	AT →	CG	TV
→	UCA	(SER)	AT →	CG	TV
→	UGA	(OPAL)			
→	UAG	(AMBER)			

TV - Transversion

TI - Transition

leucine replacement GDH enzymes were shown to be distinguishable from each other and from wild-type by thermostability measurements at two pH values, and by their requirement for high concentrations of both glutamate and NADP in order to maintain an active conformation. The effect of this latter property is to markedly decrease the enzyme activity in assay system C (Coddington _et al._, 1966) which measures glutamate oxidation. It should be possible to characterise some, if not all, of the replacements in any NQO-induced _am 17_ revertants by comparing their activities in enzyme assays with those of the three characterised revertants plus wild-type.

As well as studying the specificity of NQO in this way it should also be possible to use the same system to investigate the specificities of three other mutagens; UV light, nitrous acid, and EMS. The specificities of these mutagens has already been discussed in the introduction to the thesis where it was pointed out that little has been published about their specificity in _Neurospora_.

As well as these reversion studies with _am 17_ it is also possible to investigate the specificities of the four mutagens NQO, UV, nitrous acid and EMS by using three other mutants; _am 1_, _am 3_ and _am 7_. These three mutants were characterised by amino-acid sequencing studies and their base-pair alterations deduced (Brett _et al._, 1976). The mutants _am 1_ and _am 7_ both require an AT → GC transition to revert to wild-type, while the mutant _am 3_ requires a GC → AT transition to revert to wild-type (see Fig. 1). Using these mutants it is possible to test the ability of the four mutagens to induce both types of transition. However there is an added complication in reversion studies using the mutant _am 3_. There appear to be a number of sites within the

am gene which can mutate so as to partly suppress the effect of the *am 3* mutation on enzyme activity (Pateman and Fincham, 1965). Presumably in these instances a second amino-acid change can restore the GDH activity absent in the *am 3* mutant. Thus in order to demonstrate the ability of a mutagen to induce GC → AT transitions of *am 3* it is necessary to identify true wild-types amongst the revertants by using enzyme assays.

These studies should tell us something about the mutagenic specificity of the mutagens NQO, UV, EMS and nitrous acid in the organism *Neurospora crassa*, and hopefully should provide useful clues as to the identity of the *am 17* nonsense codon.

ii) MATERIAL AND METHODS

1 (a) MEDIA

All media consisted of 1) inorganic salt solution 2) a carbon source. 1.5% DIFCO agar was added to form a solid substrate.

1) Two types of salt solution were used:

a) Westergaard's salts (2x)

b) Vogel's salts (50x)

a) Westergaard's salts (2x) 1 litre. pH 6.5

H_2O	1000 mls
KNO_3	2 g
KH_2PO_4	2 g
$MgSO_4\ 7H_2O$	1 g
NaCl	0.2 g
$CaCl_2\ 2H_2O$	0.2 g
Biotin stock	1 ml
Trace elements	0.5 mls
Chloroform	1-2 mls

Biotin stock	100 mls
Biotin	1 mg
H_2O	50 ml
Ethanol	50 ml

Westergaard's Trace Elements	250 mls
H_2O	250 mls
Na_3BO_3	0.01 g
$Cu\ SO_4\ 5H_2O$	0. 1 g
$Fe\ PO_4$	0. 2 g
$Mn\ SO_4\ H_2O$	0.02 g
$Na\ Mo\ O_4\ 2H_2O$	0.02 g
$Zn\ SO_4\ 7H_2O$	2 g

b) Vogel's salts (50x) 1 litre

H_2O	750 mls
Na_3 citrate	150 g
KH_2PO_4	250 g
NH_4NO_3	100 g
$MgSO_4 \cdot 7H_2O$	10 g
$CaCl_2$ (dissolved in 100 mls H_2O)	5 g
Biotin stock	12.5 mls
Trace elements	5 mls
Chloroform	1-2 mls

Vogel's Trace Elements 100 mls

H_2O	95 mls
Citric acid	5 g
$ZnSO_4 \cdot 7H_2O$	5 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	1 g
$CuSO_4 \cdot 5H_2O$	0.25 g
$MnSO_4 \cdot H_2O$	0.05 g
H_3BO_3	0.05 g
$NaMoO_4 \cdot 2H_2O$	0.05 g

2) CARBON SOURCES

2% sucrose was used as the carbon source with tube and flask cultures. If colonial growth was required on plates then 2% sorbose was used with 0.2% sucrose.

The two basic media used were 1) Vogel's minimal medium 2) Westergaard's crossing medium.

1) Vogel's Minimal Medium 1 litre

Vogel's salts (x50)	20 mls
Sucrose	20 g
Agar	15 g
H_2O	980 mls

2) Westergaard's Crossing Medium — 1 litre

Westergaard's salts (2x)	500 mls
Sucrose	20 g
Agar	15 g
H_2O	500 mls

Supplements

Glutamate	0.5 mg ml^{-1}
Glycine	1.5 mg ml^{-1}

1) 0.1 M phosphate, EDTA

For one litre

	0.2M Na_2 HPO_4	0.2M Na H_2 PO_4 $2H_2O$	H_2O
pH 6.5	160 mls	340 mls	500 mls
pH 7.4	405 mls	95 mls	500 mls
pH 8.0	473.5 mls	26.5 mls	500 mls

Solutions are made 1 mM EDTA

(b) MUTAGENS

NQO - 4-Nitroquinoline-1-Oxide (Sigma), dissolved in DMSO before adding to conidial suspension to a limit of 5% DMSO

UV - Ultraviolet light, provided by a low pressure mercury vapour lamp (Hanovia No. 772/64) with approximately 85% of output at the 254 nm wavelength. Irradiation was from 10 cm.

NA - Nitrous acid, 5mM sodium nitrite (Na NO_2) in 0.1M acetate buffer pH 4.5.

EMS - Ethyl methane sulphonate, C_2H_5 OSO_2 H_3 (Sigma)

(c) MAINTENANCE OF STOCKS

Short term storage of Neurospora strains on a week to week basis was on 1 ml Vogel's minimal slants with appropriate supplements.

Strains were transferred to slants either as conidia on wet wire loops, or as colonies cut out of agar plates. Freshly innoculated slants were incubated at 25°C for approximately 7 days.

Long term storage of strains was as silica gel stocks. To make these, a conidial suspension in sterile distilled water was added, on ice, to dehydrated silica gel crystals in a 5 ml test tube. The crystals take up the water leaving the dry conidia attached to the crystal surfaces. The stocks are kept at 5°C.

To start a culture from silica gel stocks requires the tipping of several crystals onto a suitable medium.

(d) REVERSION ANALYSIS

1) GROWTH AND HARVESTING

The mutant strain to be tested was grown up on a Vogel's minimal plus glutamate flask slant. The conidia were harvested by adding approximately 20 mls 0.1M phosphate buffer pH 7.0 and shaking vigorously. The conidial suspension was filtered through cotton wool to remove debris and then spun down at 2000 rpm and washed twice before being resuspended in 20-50 mls of buffer to a concentration of 1-5 x 10^{7} ml^{-1} as measured on a haemacytometer.

2) MUTAGEN TREATMENT

(a) NQO: NQO was dissolved in DMSO and added to conidial suspension at 30°C. The final concentration of DMSO was no more than 5%. At the end of 2 hours treatment conidia were filtered and washed with 10% sodium thiosulphate through a Buchner funnel and membrane filter (OXOID)apparatus before being resuspended in buffer.

(b) EMS: The procedure was the same as for NQO except that EMS is a liquid and was added directly to the conidial suspension. Treatment was for 40 minutes at 30°C.

(c) UV: the conidia were irradiated in solution in petri dishes while being stirred with a magnetic stirrer at room temperature.

(d) NA: conidia were filtered through a suction apparatus and resuspended in nitrous acid solution

at 30°C. At the end of treatment the conidia were suction filtered and washed with 10% sodium thiosulphate solution. They were resuspended in buffer.

Treated conidia were plated out to measure reversion frequency and survival. The medium used to measure reversion frequency was Vogel's sorbose minimal medium supplemented with glycine. _am_ mutants are able to grow on minimal medium albeit, after some delay, at a slower rate than wild-type. Glycine in the medium helps to minimise this 'leakiness'. Even with glycine the conidia have to be plated at low densities so that growth does not overwhelm any revertants. Conidia were plated out by adding them to molten medium kept at 45°C and poured into petri dishes to a concentration of no more than 5 x 10^5 conidia per plate (containing 25-30 mls medium).

To measure survival, the treated conidia were diluted in distilled water and plated out in Vogel's sorbose plus glutamate medium to give no more than 200 viable conidia per plate.

After the plates were set they were incubated at 25°C.

Revertant colonies would begin to appear after about 3 days, were scored after 5 days, and picked onto Vogel's minimal plus glycine slants. Conidia produced by revertants were further tested by inoculating them into Vogel's liquid minimal plus glycine and comparing their growth with that of the original mutant and wild-type.

3) BACKCROSSING REVERTANTS

Neurospora has two mating types, _A_ and _a_. For a cross to be successful the parent strains have to be of the opposite mating type.

Crosses were set up by inoculating Westergaard's minimal plus glutamate slants with an _am_ mutant of opposite mating type to the one under investigation. Westergaard's medium

favours the production of the female sexual organs or protoperithecia after about 7 days of inoculation at 25°C. After about 10 days conidia from the revertants were suspended in distilled water and poured onto the slants. The conidia fertilised the protoperithecia which within a few days blackened and enlarged to become perithecia, or fruiting bodies. Almost 7 days after fertilisation, these perithecia began to eject spores onto the far side of the tube from where they were picked off with a wet wire loop and spread on Vogel's sorbose minimal plus glycine medium. The spores were heat-shocked at 60°C for 40 minutes to induce germination. After about 48 hours at 25°C the revertant sporelings were ready to be picked off onto Vogel's minimal plus glycine slants.

Backcrossing was carried out for two purposes: to segregate out unwanted secondary mutations and to get rid of any non-revertant nuclei which can persist in revertant mycelium through heterokaryosis.

(e) ENZYME ASSAYS (Coddington et al., 1966).

1) GROWTH AND HARVESTING OF MYCELIUM

To produce a sufficient dry weight of mycelium for carrying out enzyme assays, 50 mls of liquid Vogel's minimal medium was inoculated, in a 250 ml flask, with a wet loop of conidia and incubated at 25°C for approximately 40 hours. The resulting mycelial pads were then filtered and washed with distilled H_2O on a suction filter and dried overnight in a freeze drying apparatus. The dried pads were stored at -20°C until required. This procedure usually yielded approximately 250 mg dry weight of mycelium.

2) PREPARATION OF CRUDE PROTEIN EXTRACTS

Approximately 50 mg of dry mycelium was powdered in a plastic Sorvall centrifuge tube by inserting two stainless steel spatulas and vibrating on a Whirlimixer. The powdered mycelium was then suspended in 2 mls of 0.1 M phosphate, 1 mM EDTA buffer of the required pH and left

on ice for 30 minutes with occasional agitation. At the end of this time the suspension was centrifuged at 6000 rpm for 5 minutes. The supernatant was removed and left on ice until required.

3) GLUTAMATE DEHYDROGENASE ASSAY SYSTEMS (Coddington *et al.*, 1966)

In vivo GDH catalyses the formation of glutamate from α-ketoglutarate plus ammonium ions with the concomitant oxidation of NADPH.

$$NH_4^+ + \alpha\text{-ketoglutarate} + NADPH \rightleftharpoons \text{Glutamate} + NADP^+ + H_2O$$

Assay system A follows this reaction by measuring the decrease in concentration of NADPH in a spectrophotometer. NADPH absorbs at a wavelength of 340 nm.

In vitro, GDH can also catalyse the reverse reaction which involves the oxidative deamination of glutamate and the reduction of NADP. The assay system C measures the increase in concentration of NADPH at a wavelength of 340 nm.

The specific activities of GDH in these assays can be calculated by measuring the concentration of protein in the protein extracts used in the assays by means of the micro-biuret protein estimation procedure. Specific activities are expressed as initial change in optical density at 340 nm per minute per milligram of protein.

a) ASSAY SYSTEM A

Solutions		
(a)	0.2 M Na α-ketoglutarate (pH 6.5-7.0)	0.15 ml
(b)	1 M NH_4 Cl	0.1 ml
(c)	0.1 M Tris/HCl pH 8.5	2.55 ml
(d)	0.1% $NADPH_2$ (in Tris/HCl pH 8.5)	0.2 ml

The assay was carried out at 35°C, 50 ul of protein extract were added to the reaction mixture in a 1 cm cuvette and the change in optical density followed in a spectrophotometer at 340 nm.

The Na α-ketoglutarate and $NADPH_2$ was made up fresh.

b) ASSAY SYSTEM C

Solutions (a) 0.16 M Na glutamate (in 0.1M Tris/HCl pH 8.5)
(b) 0.2% NADP (Fresh)

50 ul of enzyme extract were added to 2.8 mls of the glutamate solution at 35°C in a 1 cm cuvette and left for 30 seconds before 0.2 mls NADP was added. Change in optical density was followed at 340 nm.

c) THERMOSTABILITY ASSAYS (Seale *et al.*, 1977)

Extracts were heated for varying periods of time at 60°C and cooled on ice before being assayed, using systems A and C. Extracts were prepared at different pH values to test whether thermostability was pH-dependent.

d) MICROBIURET PROTEIN ESTIMATION

Microbiuret reagent solution was made by adding 40 mls of 1% Cu SO_4 (in H_2O) dropwise to 150 mls of 40% Na OH (in H_2O). The solution was filtered through glass wool and stored in the refrigerator in a plastic bottle.

To calibrate the microbiuret reagent a solution of 1 mg ml^- bovine serum albumin was made up in H_2O. A series of dilutions in H_2O in a total volume of 1 ml were made and 0.5 ml of reagent added. After 20 minutes at room temperature the absorbances of the protein solutions were measured at 310 nm in a double-beam spectrophotometer against 1 ml H_2O plus 0.5 ml microbuiret reagent. The absorbances were plotted against protein concentration to obtain a calibration curve.

Protein estimations of crude protein extracts were made using 50 ul of extract in 0.95 ml H_2O plus 0.5 ml microbiuret reagent, following the procedure of the calibration experiments, and using the calibration curve to calculate protein concentration.

(iii) RESULTS

(a) REVERSION ANALYSIS OF am 1, am 3 and am 7 with NQO, UV, NA and EMS.

1. NQO

Table 2.1 gives the results of the treatment of the three mutants with NQO.

The mutant am 7 which requires an AT → GC transition to revert is almost non-revertible with NQO even at levels of survival as low as 2%.

However, the am 1 mutant, which also requires an AT → GC transition to revert, does revert with NQO up to a reversion frequency of 1.8×10^{-6} surviving conidia.

The am 3 mutant, which requires a GC → AT transition to revert to wild-type, shows the best response to NQO of all the mutants, being able to revert up to a frequency of 8.6×10^{-6} surviving conidia.

2. UV light

Table 2.2 gives the response of the mutants to UV treatment.

UV is able to revert all of the three mutants. The am 7 mutant reverts up to a frequency of 1.0×10^{-6} surviving conidia, while am 1 is able to revert at a higher frequency of 2.3×10^{-6} surviving conidia. The am 3 mutant shows the best response being revertible up to 9.6×10^{-6} surviving conidia.

3. Nitrous Acid

Nitrous acid was found not to be a particularly potent mutagen as far as these three mutant strains are concerned, as can be seen from the results in Table 2.3.

TABLE 2.1 NQO Reversion Analysis of *am 1*, *am 3* and *am 7*

am 1

Dose/μg ml^{-1}	% Survival	Revertants	Revertants per 10^6
0	100	0	-
0.1	98	0	-
0.2	59	3	0.4
0.4	33	2	0.2
0.6	21	4	0.7
1.5	13	3	1.8

am 7

Dose/μg ml^{-1}	% Survival	Revertants	Revertants per 10^6
0	100	0	-
0.15	84	0	-
0.25	95	0	-
0.5	91	2	<0.1
0.75	51	0	-
1.0	54	1	<0.1
1.5	26	0	-
3.0	2	0	-

am 3

Dose/μg ml^{-1}	% Survival	Revertants	Revertants per 10^6
0	100	0	-
0.25	63	15	0.9
0.5	24	28	4.6
1.0	3	6	8.6

TABLE 2.2. UV Reversion Analysis of am 1, am 3 and am 7

am 1

Dose/secs	% Survival	Revertants	Revertants per 10^6
0	100	0	-
15	88	6	0.3
20	73	15	0.6
30	53	20	1.1
45	18	3	1.6
60	6	3	2.3

am 7

Dose/secs	% Survival	Revertants	Revertants per 10^6
0	100	0	-
15	76	0	-
30	64	22	0.8
45	57	24	1.0
60	10	1	0.2

am 3

Dose/secs	% Survival	Revertants	Revertants per 10^6
0	100	0	-
15	75	5	0.6
30	44	5	1.0
45	21	16	6.8
60	13	14	9.6

TABLE 2.3. NA Reversion Analysis with am 1, am 3 and am 7

am 1

Dose/mins	% Survival	Revertants	Revertants per 10^6
0	100	0	-
20	83	2	0.2
30	62	1	0.1
40	44	1	0.2

am 7

Dose/mins	% Survival	Revertants	Revertants per 10^6
0	100	0	-
20	100	2	<0.1
35	38	2	0.2
50	16	0	0

am 3

Dose/mins	% Survival	Revertants	Revertants per 10^6
0	100	0	-
13	99	0	-
23	62	0	-
31	37	0	-
41	19	0	-

Low reversion frequencies were observed with am 1 and am 7 with a maximum of 0.2×10^{-6} surviving conidia for both strains.

The mutant am 3 was, perhaps surprisingly, found to be non-revertible with nitrous acid under the treatment conditions used.

4. EMS

The response of the three mutants to EMS treatment is presented in Table 2.4.

The two mutants requiring AT → GC transitions, am 1 and am 7, were non-revertible with EMS.

However the am 3 mutant, which requires a GC → AT transition to revert to wild-type, showed a very good response with EMS and reverted at a frequency of 40.0×10^{-6} surviving conidia at the low survival of 2%.

(b) Enzyme Analysis of NQO, UV and EMS-Induced Revertants of am 3

Although the three mutagens NQO, UV and EMS all reverted the mutant am 3 at a relatively high frequency it was not possible to say what proportion of revertants represented GC → AT transitions to wild-type without performing enzyme assays on the revertant strains. In an attempt to distinguish non-wild-type revertants from true wild-type revertants thermostability tests were performed on crude protein extracts prepared from the revertant strains. These tests consisted of incubating the extracts at 60°C for varying periods of time before subjecting them to enzyme assays.

To begin with a total of eighteen NQO, eleven UV and fourteen EMS-induced revertants were assayed for thermostability at 60°C and pH 6.5 using assay system C which measures glutamate oxidation. The results are given in Tables 2.5, 2.6 and 2.7 for the three mutagens respectively,

TABLE 2.4. EMS Reversion Analysis of am 1, am 3 and am 7

am 1

Dose/%	% Survival	Revertants	Revertants per 10^6
0	100	0	-
1	88	0	-
2	54	0	-
5	10	0	-

am 7

Dose/%	% Survival	Revertants	Revertants per 10^6
0	100	0	-
1	76	0	-
2	61	0	-
5	20	0	-

am 3

Dose/%	% Survival	Revertants	Revertants per 10^6
0	100	0	-
1	75	8	2.4
2	34	56	8.3
5	2	16	40.0

TABLE 2.5. Heat Stability Assay of NQO-Induced *am 3* Revertants.

Using assay C after heating extracts for 0, 10 and 20 minutes at 60°C and pH 6.5

Strain	Log % Initial Activity		
	0 Mins	10 Mins	20 Mins
STa	2.00	1.57	1.30
N1	2.00	1.53	1.28
N2	2.00	1.60	1.33
N3	2.00	0.30	0
N4	2.00	0.79	0
N6	2.00	0.34	0
N8	2.00	1.56	1.24
N9	2.00	1.22	0.88
N10	2.00	1.40	0.92
N11	2.00	1.64	1.22
N12	2.00	0.85	0
N13	2.00	1.66	1.38
N14	2.00	0.66	0
N15	2.00	1.52	0.96
N17	2.00	0.79	0
N18	2.00	0	0
N19	2.00	1.64	1.40
N20	2.00	1.56	1.22
N21	2.00	1.48	1.21

TABLE 2.6. Heat Stability Assay of UV-Induced *am 3* Revertants

Using assay C after heating extracts for 0, 5 and 10 minutes at 60°C and pH 6.5.

Strain	Log % Initial Activity		
	0 Mins	5 Mins	10 Mins
STa	2.00	1.75	1.61
U1	2.00	0	0
U2	2.00	1.77	1.56
U3	2.00	1.71	1.56
U4	2.00	1.78	1.65
U7	2.00	1.79	1.70
U9	2.00	1.74	1.50
U11	2.00	1.79	1.72
U14	2.00	1.84	1.79
U15	2.00	1.70	1.63
U18	2.00	1.72	1.69
U19	2.00	1.80	1.55

TABLE 2.7. Heat Stability Assay of EMS-Induced *am 3* Revertants

Using assay C after heating extracts for 0, 15 and 30 minutes at 60°C and pH 6.5.

Strain	Log % Initial Activity		
	0 Mins	15 Mins	30 Mins
STa	2.00	1.47	0.96
E1	2.00	1.20	0.58
E2	2.00	1.50	1.09
E3	2.00	1.25	0.59
E5	2.00	1.16	0.59
E6	2.00	1.55	1.07
E8	2.00	1.49	1.09
E9	2.00	1.43	0.97
E10	2.00	1.46	1.03
E11	2.00	0	0
E12	2.00	1.66	1.21
E13	2.00	1.49	0.89
E14	2.00	1.41	0.87
E15	2.00	1.16	0.43
E16	2.00	1.64	1.20

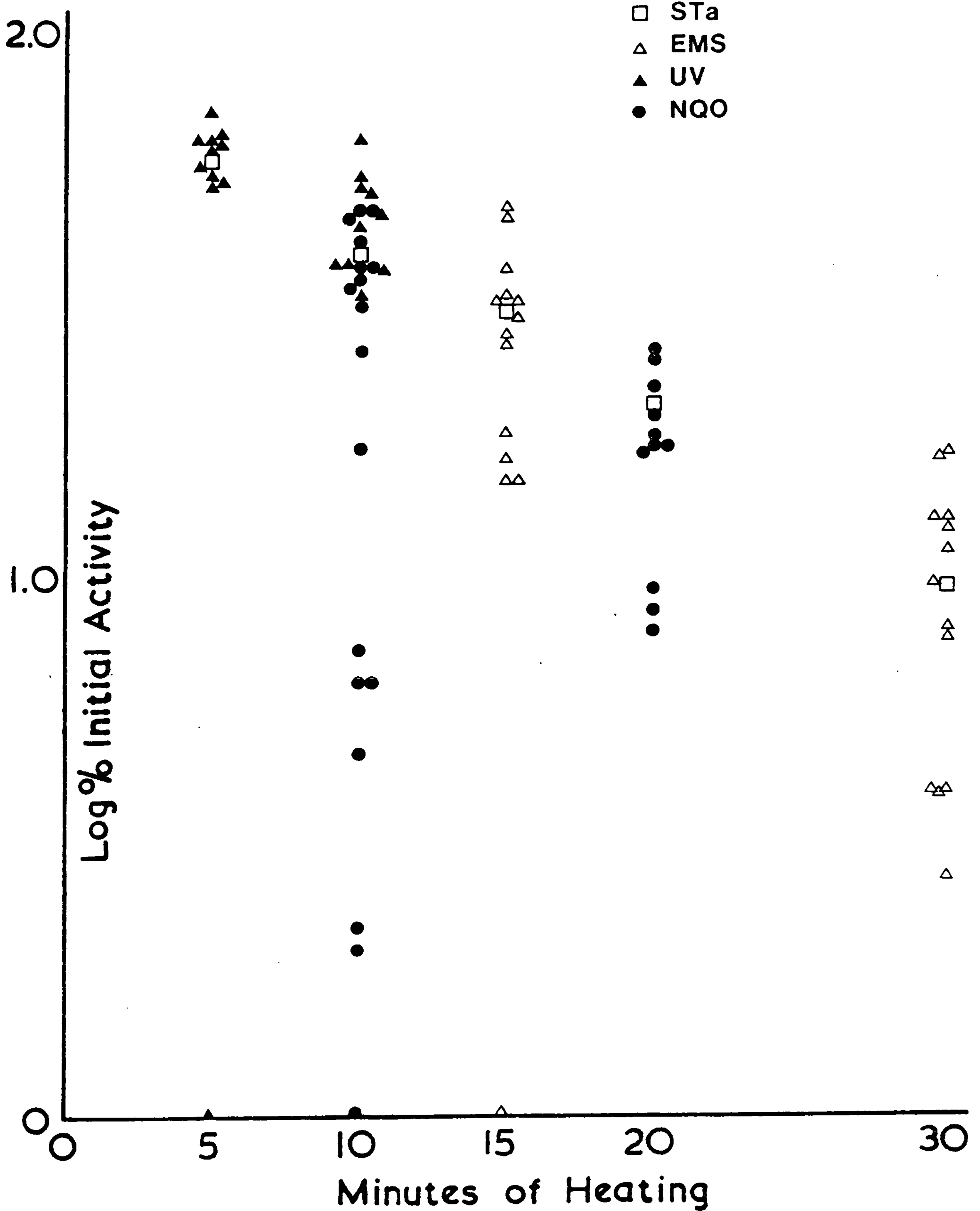


Fig. 2.2 Thermostability Assay Of UV, EMS & NQO-Induced <u>am 3</u> Revertants At pH6.5 Using Assay C.

and are plotted together in Figure 2.2.

The results show that the majority of the revertants show wild-type stability to heat inactivation under these conditions. These revertants were then further assayed for thermostability at 60°C but this time at pH 8.0 and using assay system A which measures glutamate synthesis. The results are presented in Table 2.8 and are plotted in Figure 2.3.

On the basis of these results the revertants, N20, U4, U7, U9, U11, U15, U18, U19, E2, E8 and E10 are distinguishable from wild-type.

The specific activities, calculated as change in optical density per minute per milligram of protein, of the revertants which showed any activity at pH 8.0 were measured and are given in Table 2.9. From these results it can be seen that five of the NQO-induced revertants (N1, N8, N11, N19 and N21), one of the UV-induced revertants (U2), and four of the EMS-induced revertants (E6, E9, E13 and E14) have specific activities within 50% of wild-type activity, as well as showing wild-type stability to heat inactivation.

These observations are consistent with the expectation that all three mutagens should be able to induce the GC → AT transition required to revert am 3 back to wild-type.

However it is very clear that enzyme assays are unable to unambiguously identify wild-type revertants. Some doubt must inevitably remain as to whether the five NQO, one UV and four EMS-induced revertants listed above actually represent wild-type revertants.

It is clear from these studies that it is possible to obtain a large number of revertant classes from reversion studies with am 3. Pateman and Fincham (1965) were able to identify six classes of revertant from studies of thirteen UV-induced revertants of am 3. Any estimation of GC → AT transition frequency made from reversion studies with am 3 represents a minimum estimation as some of the non-wild-

TABLE 2.8. Thermostability of NQO, UV and EMS-Induced am 3 Revertants at pH 8.0 in Assay System A

Strain	Log % Initial Activity			
	0 Mins	10 Mins	20 Mins	at 60°C
STa	2.00	1.79-1.91	1.58-1.73	
N1	2.00	1.97	1.88	
N2	2.00	1.81	1.52	
N8	2.00	1.79	1.52	
N11	2.00	1.73	1.39	
N13	2.00	2.22	2.05	
N19	2.00	1.86	1.63	
N20	2.00	1.57	1.11	
N21	2.00	1.72	1.36	
U2	2.00	1.72	1.45	
U3	2.00	1.75	1.45	
U4	2.00	1.63	1.20	
U7	2.00	1.38	0.70	
U9	2.00	1.76	1.48	
U11	0			
U15	0			
U18	0			
U19	0			
E2	2.00	1.46	0.79	
E6	2.00	1.78	1.59	
E8	0			
E9	2.00	1.79	1.59	
E10	0			
E13	2.00	1.82	1.63	
E14	2.00	1.76	1.58	

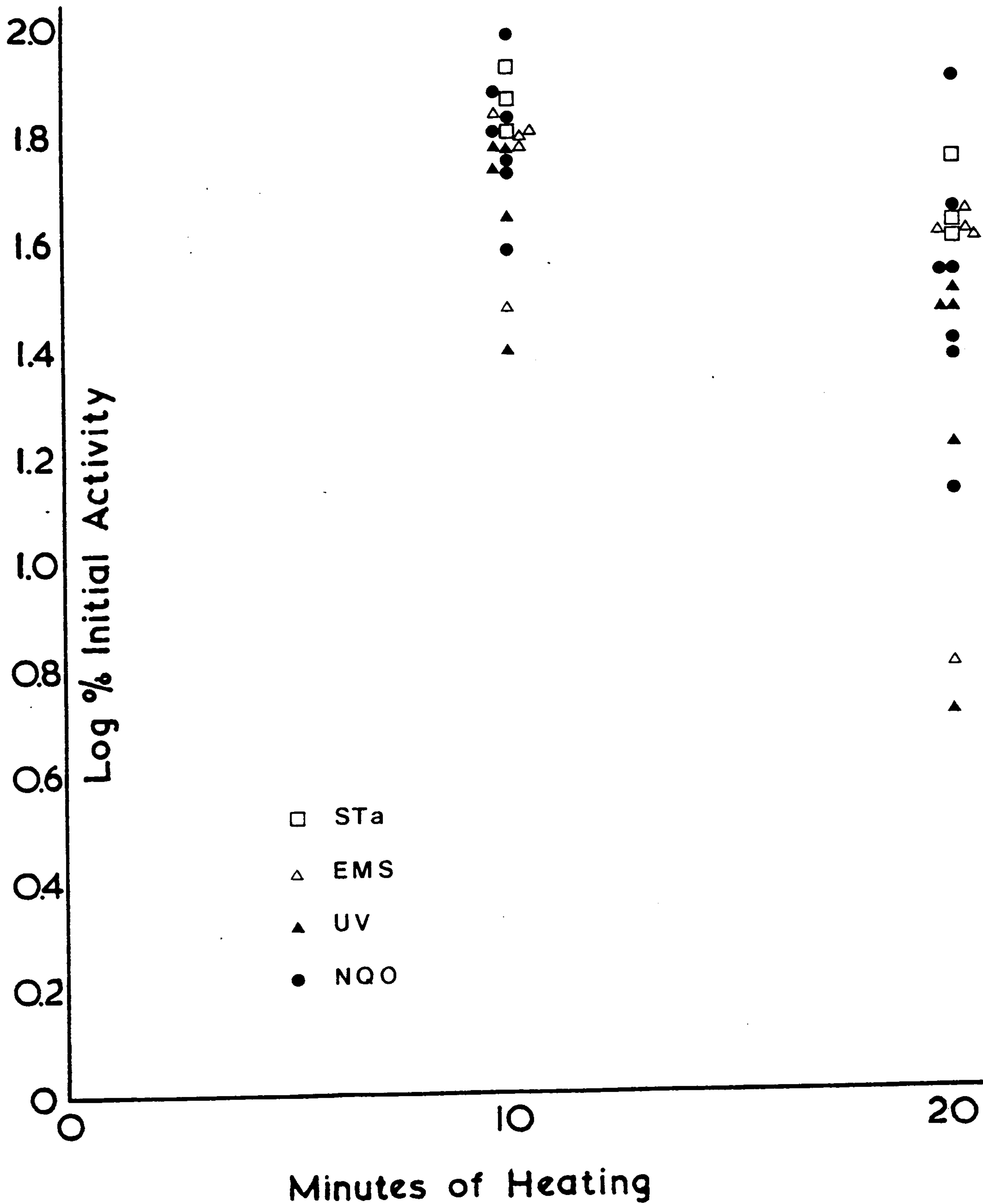


Fig. 2.3 Thermostability Of NQO, UV & EMS-Induced am 3 Revertants At pH8.0 In Assay System A.

TABLE 2.9. Specific Activity of NQO, UV and EMS-Induced Revertants in Assay System A, Expressed as Percentage of Wild-Type Activity.

Strain	Assay A %
N1	111
N2	32
N8	87
N11	64
N13	20
N19	58
N20	31
N21	118
U2	146
U3	162
U4	143
U7	140
U9	183
E2	154
E6	107
E9	94
E13	86
E14	94

type revertant classes may also arise as the result of a GC → AT transition.

(c) Reversion Analysis of am 17 with NQO, UV, NA and EMS

The nonsense mutant am 17 was treated with the mutagens NQO, UV, nitrous acid and EMS. The results are presented in Table 2.10. The am 17 strain appears to be non-revertable with EMS but reverts after treatment with the other three mutagens. NQO was able to revert am 17 at a very high frequency, while UV and nitrous acid were both able to revert at a relatively low frequency.

NQO was also found to be able to revert two other am nonsense mutants; am 109 and am 142 (see Table 2.11). These two mutants were originally isolated by Kinsey (1982) and were found to be suppressed by the same supersuppressors as am 17. This strongly suggests that they represent the same class of nonsense mutation as am 17.

From the results of the reversion analyses of am 1, am 3 and am 7 it could be concluded that NQO is able to induce mutations of GC base pairs and also of AT base pairs. Although NQO appeared to induce GC transitions with no greater efficiency than AT transitions it is dangerous to draw too many conclusions from reversion frequencies at different sites within the gene. Thus although the high frequency of reversion of am 17 with NQO suggests that am 17 is an amber mutant on the basis of the known specificity of NQO in other systems, it would be unsafe to reach such a conclusion from the results of these studies without additional evidence.

If NQO reverts am 17 by inducing transversions of the GC base pair within the nonsense codon UAG (amber), then one would expect the revertants to have mainly tyrosine replacements (UAC or UAU). Alternatively, if the NQO is inducing mutations of AT base pairs within the nonsense codon UAA (ochre), then one would expect to observe no specificity in the type of replacement within the revertants.

TABLE 2.10. Reversion of am 17 with NQO, NA, UV and EMS

NQO

Dose/μg ml^{-1}	% Survival	Revertants	Reversion Frequency
0	100	0	-
0.5	97	152	5.2×10^{-6}
1.0	46	253	18.3×10^{-6}
1.5	11	153	45.0×10^{-6}

NA

Dose/Minutes	% Survival	Revertants	Reversion Frequency
0	100	0	-
20	98	15	0.5×10^{-6}
30	85	11	0.4×10^{-6}
40	45	12	0.9×10^{-6}

UV

Dose/Seconds	% Survival	Revertants	Reversion Frequency
0	100	1	$<0.1 \times 10^{-6}$
15	92	11	0.9×10^{-6}
30	83	27	2.7×10^{-6}
45	76	16	1.8×10^{-6}

EMS

Dose/%	% Survival	Revertants	Reversion Frequency
0	100	0	-
1	93	0	-
2	72	0	-
5	37	0	-

TABLE 2.11. Reversion of am 109 and am 142 with NQO

am 109

Dose/μg ml^{-1}	% Survival	Revertants	Reversion Frequency
0	100	1	$<0.1 \times 10^{-6}$
0.1	100	10	0.4×10^{-6}
0.25	63	42	2.6×10^{-6}
0.5	2	9	22.5×10^{-6}

am 142

Dose/μg ml^{-1}	% Survival	Revertants	Reversion Frequency
0	100	0	-
0.25	49	31	1.7×10^{-6}
0.5	16	41	6.8×10^{-6}
1.0	5	9	4.7×10^{-6}

These two alternatives were tested by carrying out enzyme assays on the protein extracts from several NQO induced revertants of am 17, and comparing these to four standard strains; RN35, a tyrosine replacement, RU9, a leucine replacement, am 17, ssu 1, a tyrosine tRNA super-suppressor, and wild-type.

The same assays were carried out on revertants of am 17 induced with UV and nitrous acid not only to investigate the specificities of these two mutagens, but also to show whether any observed NQO specificity was a property of the mutagen or the locus itself.

(d) Enzyme Analysis of am 17 Revertants

(1) NQO Revertants

Crude protein extracts were prepared from twenty-seven NQO induced am 17 revertants which had been backcrossed to the mutant am 1.

Enzyme assay systems A and C were used to determine the activity of GDH from the revertant strains and from the four standard strains; STa (wild-type), RN35, RU9 and am 17 ssu 1.

Activities were calculated as change in optical density per minute per milligram of protein and then expressed as a percentage of the wild-type activity. The results are presented in Table 2.12 and plotted as activity in assay C against assay A in Figure 2.4.

If the possibility of there being other revertant types apart from the four standards is ignored for the moment, and the revertants are classified simply in terms of which standard strain they resemble most, it can be seen that; Q29 resembles wild-type; Q7, Q8 and Q27 resemble RU9; Q3, Q18, Q20, Q23, Q24, Q26 and Q28 resemble am 17 suu 1; and the other sixteen revertants resemble RN35.

The considerable deviation of the revertant activities from the standard strain activities may in some part be due

TABLE 2.12. Specific Activity of NQO-Induced am 17 Revertants in Assay Systems A and C, Expressed as Percentage of Wild-Type Activity.

Strain	Assay C %	Assay A %
RU9	18.7	78.5
RN35	29.5	131.9
am 17 ssu 1	14.3	45.8
Q1	20.1	132.3
Q2	28.1	132.3
Q3	12.1	59.2
Q4	29.0	140.5
Q5	29.4	145.7
Q7	12.3	72.9
Q8	10.1	72.1
Q9	34.5	117.5
Q10	30.9	124.9
Q11	33.3	107.7
Q12	34.0	149.5
Q13	35.6	134.1
Q15	36.2	171.0
Q16	29.2	127.6
Q17	23.2	111.7
Q18	15.3	60.9
Q19	29.9	104.1
Q20	16.3	56.8
Q21	40.6	122.8
Q22	40.2	151.4
Q23	21.5	42.8
Q24	19.9	37.4
Q25	23.7	122.2
Q26	8.3	14.7
Q27	21.4	94.9
Q28	7.8	15.7
Q29	121.4	119.3

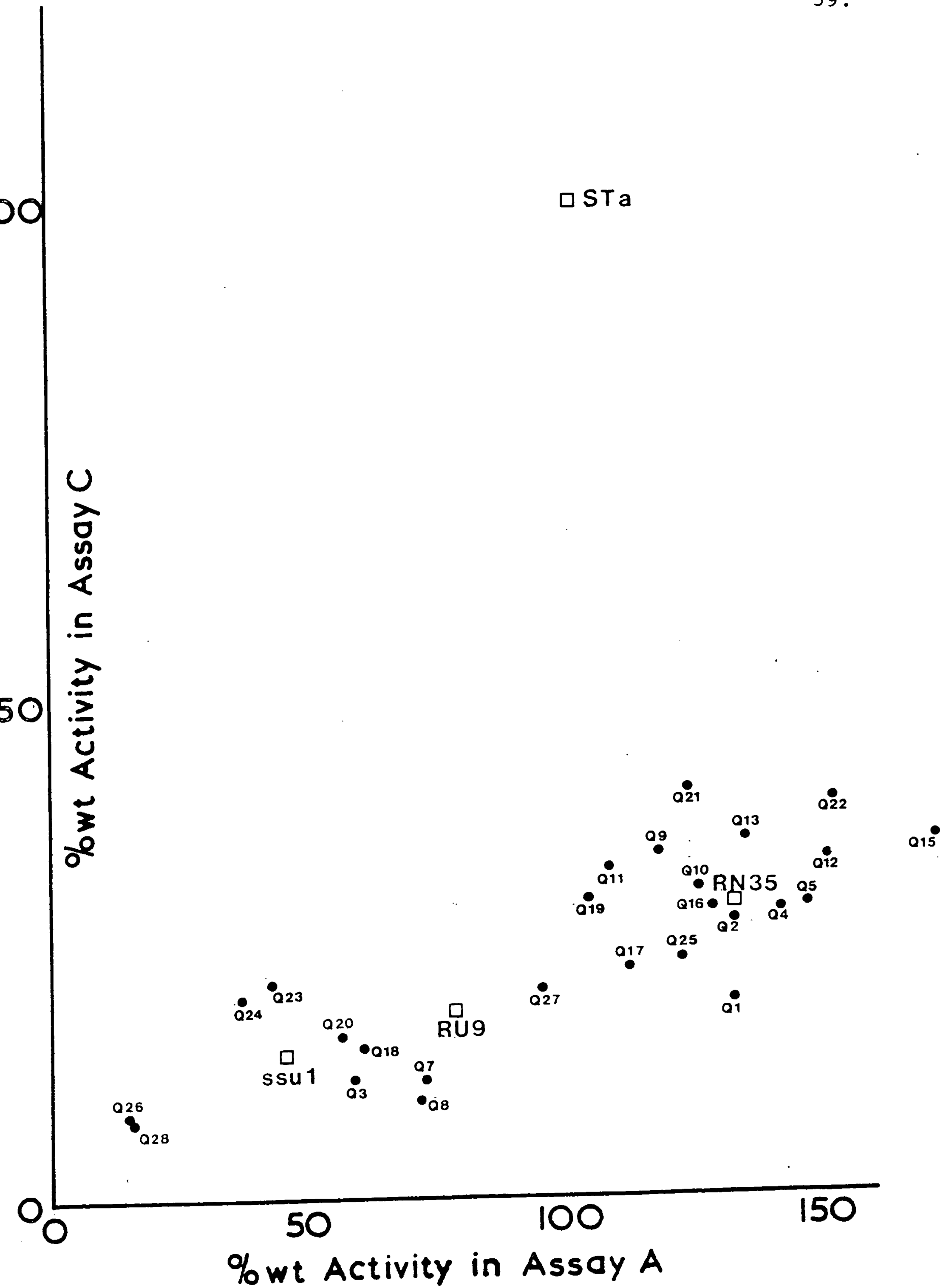


Fig. 2.4 Specific Activity Of NQO-Induced am 17 Revertants In Assay Systems A & C.

to the differing genetic backgrounds of the two groups. The standard strains are isogenic having been backcrossed to a standard wild-type strain at least six times. However the revertant strains were backcrossed at the most twice to a standard genotype. Therefore it is likely that a significant proportion of secondary mutations persist in the revertants after the first or second backcross. If these in any way affect the proportion of GDH to total cellular protein in the Neurospora mycelium then they will affect the measurement of GDH activity which is expressed in terms of total cellular protein.

In order to investigate the possibility of there being other amino-acid replacements represented within the twenty seven revertants, and to provide more data for classifying them, a thermostability assay was carried out on twenty-one of the revertants. This assay involved heating crude protein extracts from the revertants and standards at 61°C and pH 8.0 for varying periods of time before using the system A assay on the extracts. The results are presented in Table 2.13 and plotted in Figure 2.5.

Several points are clear from these results. The seven putative am 17 ssu 1 strains plus two of the putative leucine replacements, Q7 and Q8, appear to possess tyrosine replacements. Thus they are likely to resemble am 17 ssu 1 in having a mutated tyrosine tRNA gene which allows for insertion of a tyrosine residue opposite the nonsense codon.

The Q17 and Q27 revertants which lie between RU9 and RN35 in the previous activity assays, behave very clearly like RU9 in the thermostability assay. Therefore it is most likely that Q17 and Q27 represent revertants in which the nonsense codon has mutated to a leucine codon.

The Q29 revertant shows the same stability to heat inactivation as the wild-type strain and therefore most likely represents a reversion to wild-type.

The remaining nine revertants all appeared to closely

TABLE 2.13. Thermostability Assay of NQO-Induced *am 17* Revertants.

Using system A assay after heating extracts at 61°C at pH 8.0 for 0, 10, 20 and 35 minutes.

Strain	Log % Initial Activity			
	0 Mins	10 Mins	20 Mins	35 Mins
STa	2.00	2.00	1.91	1.81
RU9	2.00	1.55	1.03	0.46
RN35	2.00	1.77	1.58	1.27
am 17 ssu 1	2.00	1.72	1.59	1.24
Q3	2.00	1.80	1.59	1.34
Q5	2.00	1.80	1.58	1.33
Q7	2.00	1.75	1.54	1.24
Q8	2.00	1.79	1.59	1.28
Q9	2.00	1.81	1.60	1.23
Q11	2.00	1.75	1.58	1.27
Q12	2.00	1.80	1.60	1.34
Q13	2.00	1.78	1.55	1.30
Q15	2.00	1.83	1.62	1.36
Q17	2.00	1.62	1.26	0.48
Q18	2.00	1.84	1.64	1.30
Q19	2.00	1.80	1.60	1.29
Q20	2.00	1.72	1.55	1.24
Q21	2.00	1.78	1.59	1.34
Q22	2.00	1.80	1.58	1.28
Q23	2.00	1.81	1.58	1.30
Q24	2.00	1.81	1.61	1.29
Q26	2.00	1.78	1.63	1.35
Q27	2.00	1.59	1.07	0.49
Q28	2.00	1.79	1.59	1.21
Q29	2.00	1.97	1.90	1.83

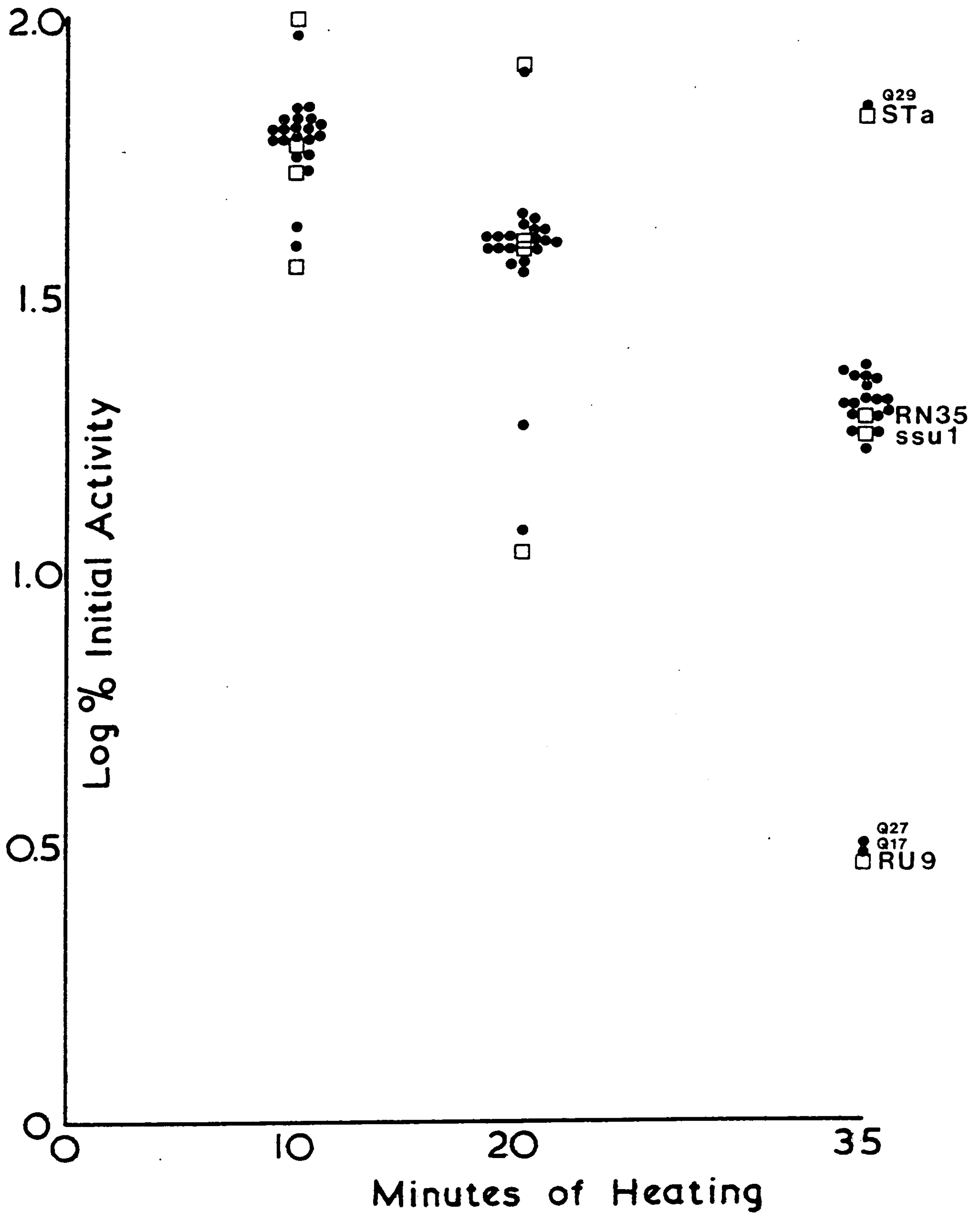


Fig. 2.5 Thermostability Assay Of NQO-Induced <u>am 17</u> Revertants.

resemble RN35 and were therefore most likely to represent mutations of the nonsense codon to a tyrosine codon.

It is worth noting that on the basis of these two sets of results there is no evidence of any other amino-acid replacement apart from the standard strains among the NQO revertants. This is not, however, to discount the possibility of there being other amino-acid replacements which behave in a similar manner to the standard strains.

One further test was carried out on the nine revertants which appear to carry supersuppressor mutations. One of the characteristics of supersuppressed mutants is that the original mutant strain can be reisolated from a back-cross to a wild-type strain. When the nine revertants, together with the am 17 ssu 1 strain, were backcrossed to wild-type it was possible to reisolate am mutants from all ten crosses. Thus the revertant strains Q3, Q7, Q8, Q18, Q20, Q23, Q24, Q26 and Q28 appear to represent super-suppressed am 17 strains.

After reclassification of the twenty-seven NQO induced revertants in the light of these further studies there now appear to be; one wild-type revertant, Q29; two leucine replacements, Q17 and Q27; nine supersuppressors (see above); and fifteen tyrosine replacements.

(2) UV Revertants

Eleven am 17 revertants induced with UV were reisolated after backcrossing to am 1. Crude protein extracts were prepared from these revertants and were assayed for GDH activity in assay systems A and C. The results are presented in Table 2.14 and plotted in Figure 2.6.

Five of the eleven revertants, V1, V4, V5, V8 and V11 resemble the wild-type strain. Two of the revertants, V6 and V10 resemble the leucine replacement strain, while another, V3, resembles the tyrosine replacement. The other three revertants are interesting as they do not appear to

TABLE 2.14. Specific Activity of UV-Induced *am 17* Revertants in Assay Systems A and C Expressed as Percentage of Wild-Type Activity.

Strain	Assay C %	Assay A %
RU9	18.7	78.5
RN35	29.5	131.9
am 17 ssu 1	14.3	45.8
V1	91.7	102.7
V2	37.7	62.5
V3	31.4	103.8
V4	88.6	93.9
V5	91.3	94.9
V6	24.3	70.7
V7	47.6	53.5
V8	86.3	94.0
V9	67.9	87.7
V10	15.0	68.5
V11	82.9	91.1

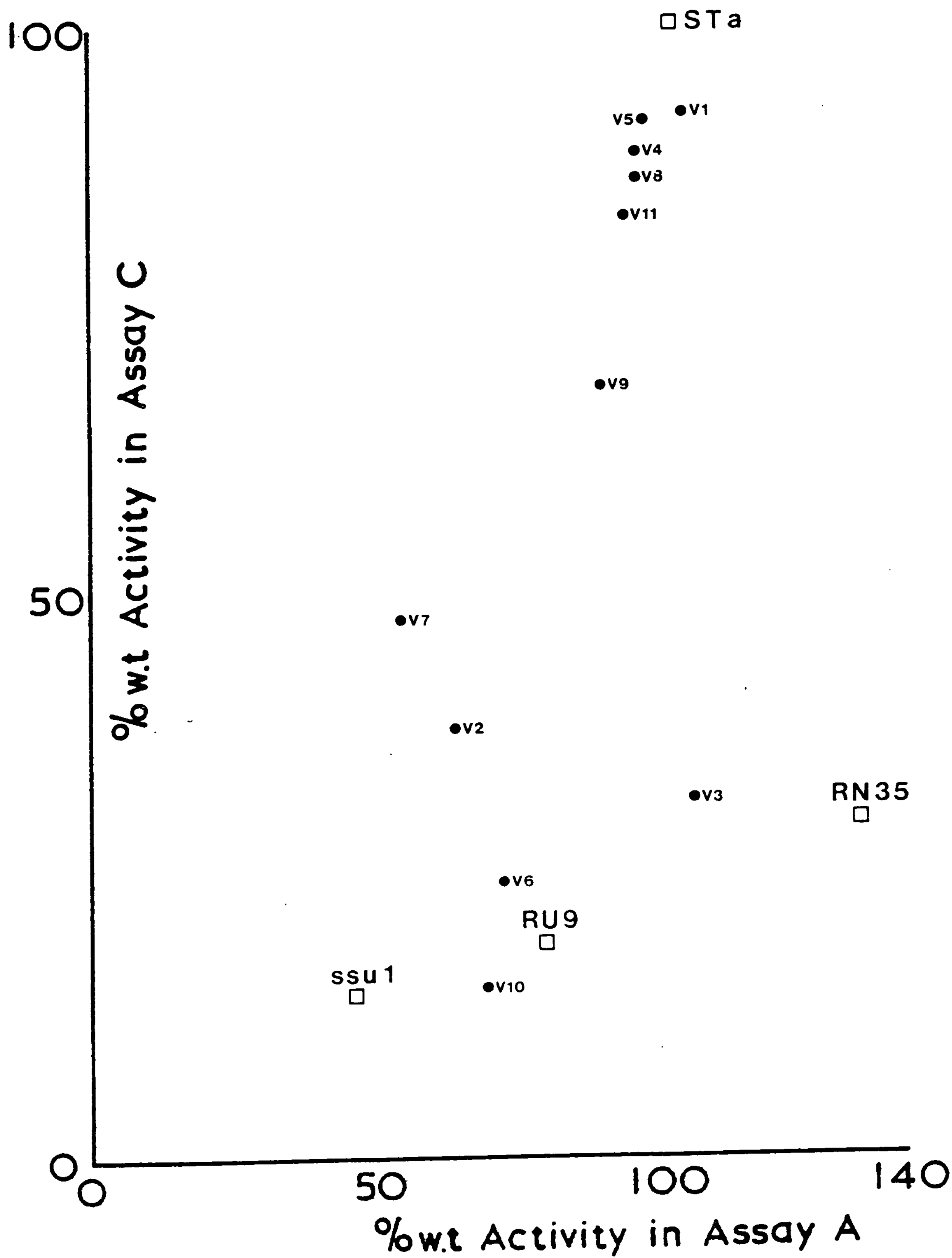


Fig. 2.6 Specific Activity Of UV-Induced <u>am 17</u> Revertants.

align with any of the standard strains. Thus V2, V7 and V9 may represent novel revertant classes.

The revertants were further analysed using the thermostability assay at pH 8.0 with assay system A. The results are presented in Table 2.15 and plotted in Figure 2.7.

The results tended to confirm the conclusions from the activity studies. Revertants V1, V4, V5, V8 and V11 can be confirmed as wild-type revertants despite their degree of resemblance to the tyrosine replacement strain in the thermostability assay. V9 also appeared to be a wild-type on the basis of its thermostability but a question mark must remain because of its low activity in the assay C system. Revertants V6 and V10 can be confirmed as leucine replacements, and V3 as a tyrosine replacement on the basis of their thermostabilities. The revertants V2 and V7 which did not behave like any of the standard strains in the activity studies again differ in the thermostability assay. They also differ from each other in the stability assay which supports the suggestion that they represent two novel classes of am 17 revertant, the V7 class being more stable than wild-type to heat inactivation, and the V2 class being extremely unstable to heat inactivation.

In another thermostability assay five of the revertants were subjected to heat inactivation at pH 6.5 before being assayed with system A. The results are presented in Table 2.16 and plotted in Figure 2.8. As expected revertants V1 displayed wild-type stability to heat inactivation at pH 6.5, while revertant V10 behaved like a leucine replacement. Of the two suspected novel classes of revertant V7 displayed wild-type activity while V2 at this pH showed no measurable activity whatsoever even without any heat inactivation.

On the basis of these results the eleven UV induced revertants of am 17 can be divided into five classes. Six revertants, V1, V4, V5, V8, V9 and V11 can be classified as wild-type revertants, V6 and V10 can be classified as leucine

TABLE 2.15. Thermostability Assay of UV-Induced *am 17* Revertants

Using system A assay after heating extracts 61°C at pH 8.0 for 0, 24 and 36 minutes.

Strain	Log % Initial Activity		
	0 Mins	24 Mins	36 Mins
STa	2.00	1.67	1.48
RU9	2.00	1.02	0.18
RN35	2.00	1.59	1.27
am 17 ssu 1	2.00	1.54	1.26
V1	2.00	1.57	1.37
V2	2.00	0.19	0
V3	2.00	1.51	1.21
V4	2.00	1.73	1.57
V5	2.00	1.76	1.56
V6	2.00	0.97	0.23
V7	2.00	1.94	1.93
V8	2.00	1.63	1.44
V9	2.00	1.70	1.55
V10	2.00	1.10	0.34
V11	2.00	1.74	1.54

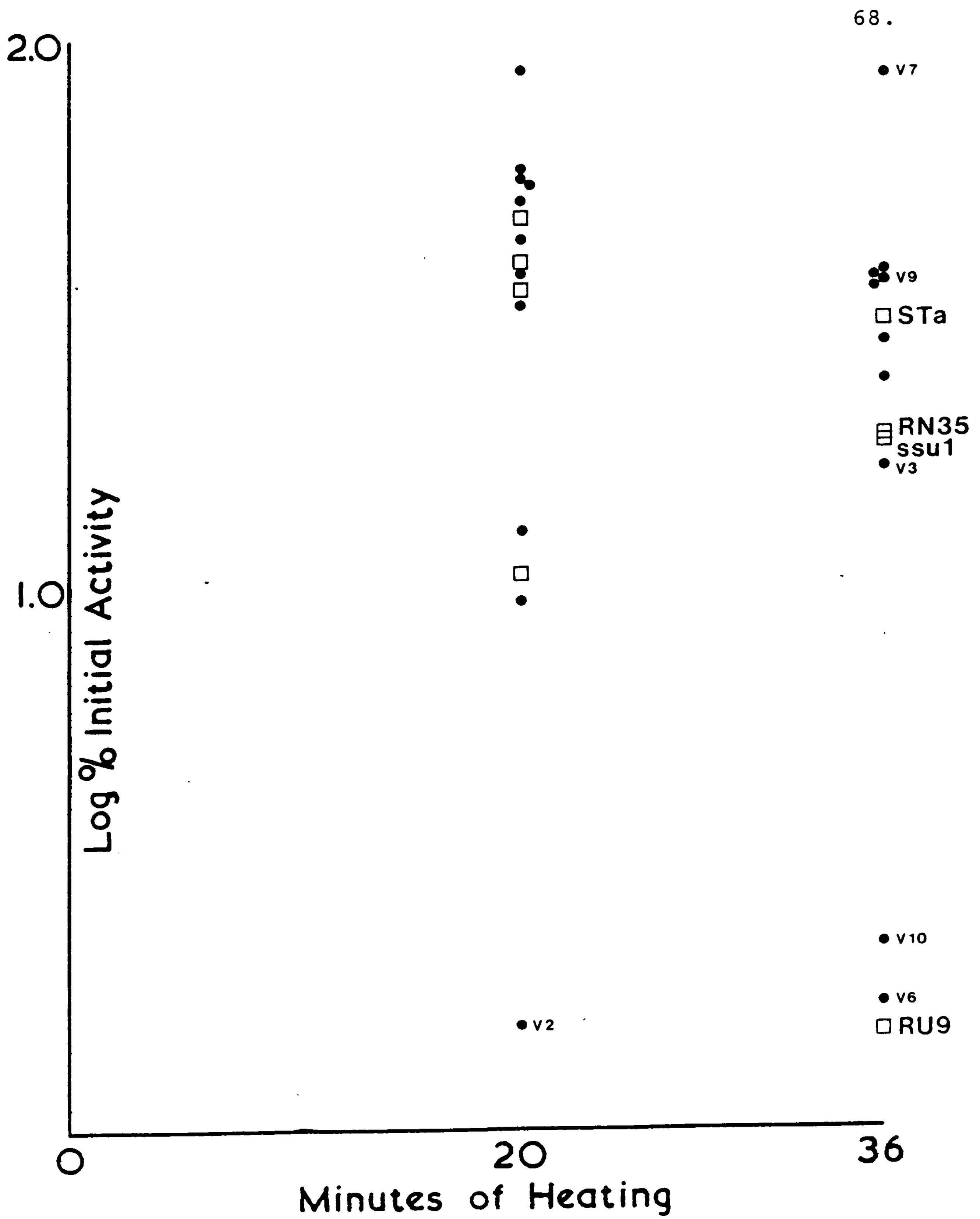


Fig. 2.7 Thermostability Assay Of UV-Induced am 17 Revertants At pH8.0 Using Assay System A.

TABLE 2.16. Thermostability Assay of UV-Induced am 17 Revertants.

Using system A assay after heating extracts at 60°C and at pH 6.5 for 0, 9½, 23 and 35 minutes.

Strain	Log % Initial Activity			
	0 Mins	9½ Mins	23 Mins	35 Mins
STa	2.00	1.65	1.34	1.00
RU9	2.00	1.62		0.80
RN35	2.00	1.84	1.61	1.40
am 17 ssu 1	2.00	1.81	1.63	
V1	2.00	1.60	1.30	0.99
V2	-	-	-	-
V5	2.00	1.70	1.28	1.02
V7	2.00	1.70	1.29	1.03
V10	2.00	1.63	1.22	0.84

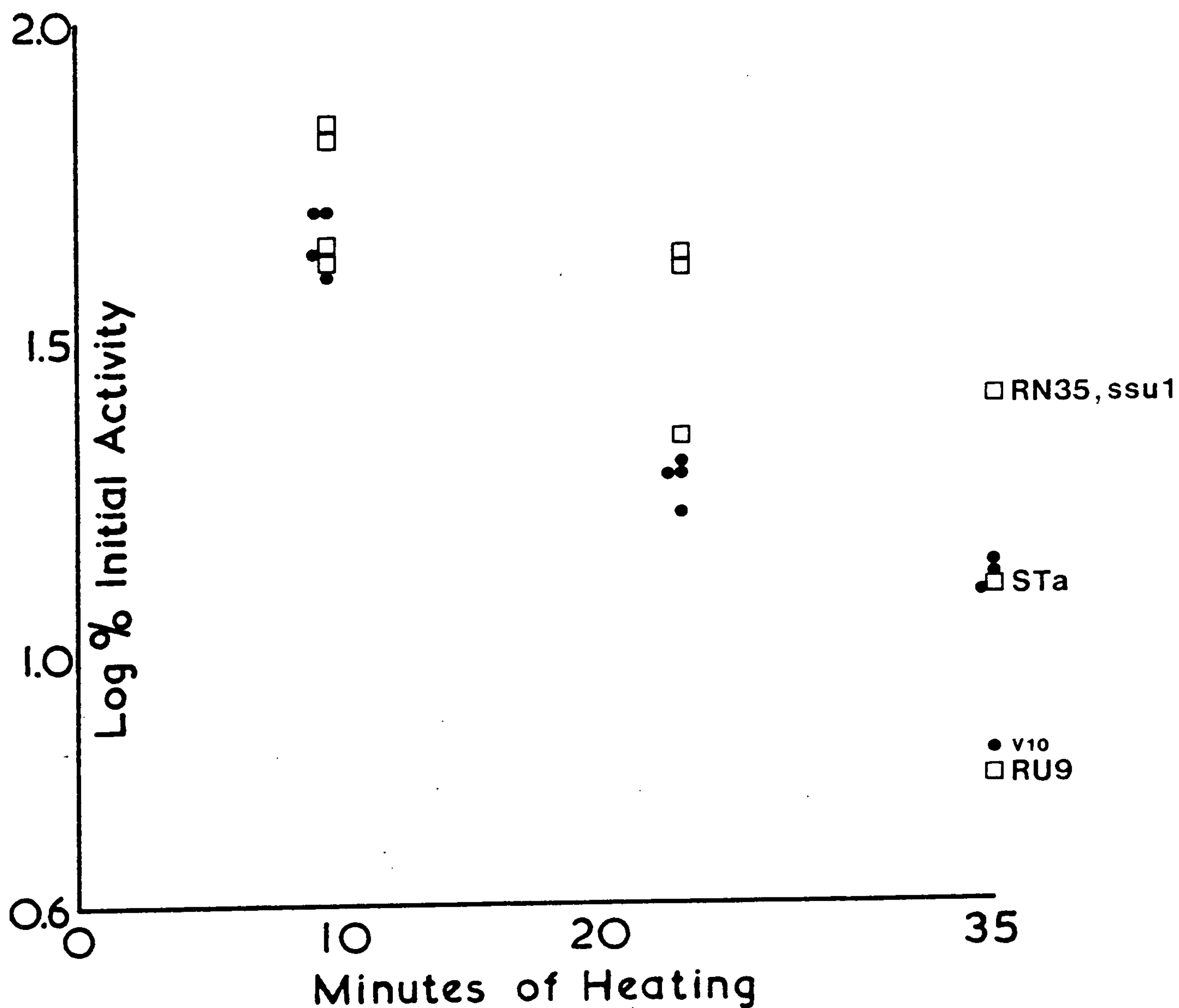


Fig. 2.8 Thermostability Assay Of UV-Induced am 17 Revertants At pH6.5 Using Assay System A.

revertants, and V3 can be classified as a tyrosine revertant. In addition to these already well-defined classes of revertant two novel classes of revertant appear to be represented among the UV-induced revertants. The revertant V2 seems to represent an amino-acid replacement which makes the GDH show approximately 50% activity in assays A and C, makes the GDH very unstable to heat inactivation at pH 8.0, and even more unstable at pH 6.5. The revertant V7 appears to represent an amino-acid replacement which also makes the GDH approximately 50% active in assays A and C, makes the GDH show the same stability to heat inactivation at pH 6.5 as wild-type, but which makes the GDH more stable than wild-type to heat inactivation at pH 8.0.

(3) Nitrous Acid Revertants

Fourteen nitrous acid induced am 17 revertants were reisolated after backcrossing to am 1. Crude protein extracts were prepared from the revertants and were assayed using systems A and C. The results are presented in Table 2.17 and plotted in Figure 2.9. None of the fourteen revertants appear to show significant departure from wild-type activity in both assays.

Crude protein extracts from twelve of the revertants were then subjected to a thermostability assay at pH 8.0 and 60°C using assay system A. The results are given in Table 2.18 and plotted in Figure 2.10.

Taken in conjunction with the activity studies these results suggest that all fourteen nitrous acid induced am 17 revertants are likely to represent wild-type revertants.

TABLE 2.17. Specific Activity of NA-Induced am 17 Revertants in Assay Systems A and C Expressed as % of Wild-Type Activity.

Strain	Assay C %	Assay A %
RU9	18.7	78.5
RN35	29.5	131.9
am 17 ssu 1	14.3	45.8
N21	98.6	85.5
N22	98.4	117.8
N23	98.8	86.4
N24	98.7	84.6
N25	99.3	109.4
N31	97.4	89.2
N32	99.3	97.0
N35	102.5	103.6
N36	100.1	88.9
N41	103.5	118.2
N42	98.1	105.2
N43	99.5	92.9
N45	94.3	107.7
N46	87.8	98.2

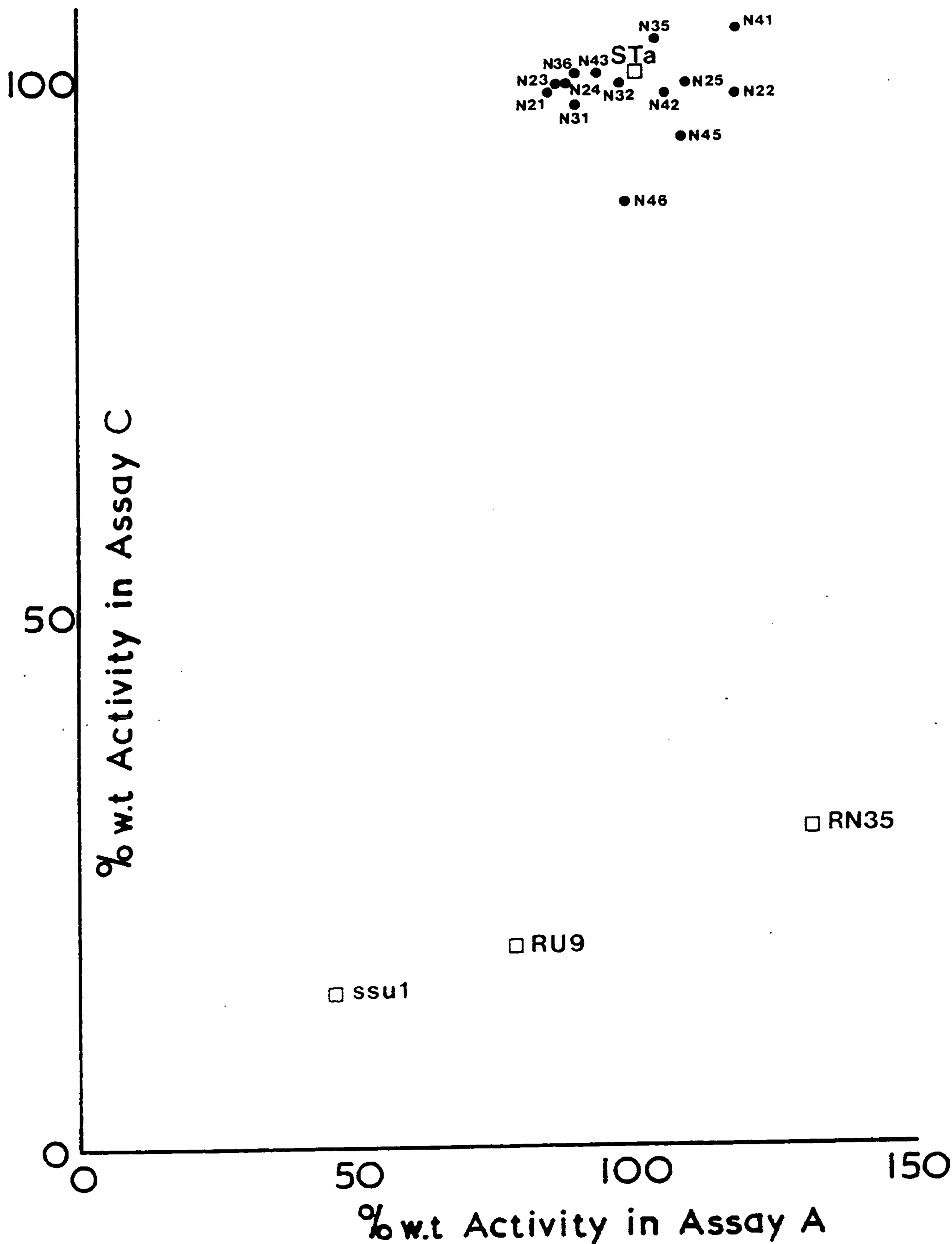


Fig. 2.9 Specific Activities Of NA-Induced am 17 Revertants In Assay Systems A & C.

TABLE 2.18. Thermostability Assay of NA-Induced am 17 Revertants

Using system A assay after heating extracts at 60°C and at pH 8.0 for 0, 20 and 35 minutes.

Strain	Log % Initial Activity		
	0 Mins	20 Mins	35 Mins
STa	2.00	1.93	1.86
RU9	2.00	1.32	1.01
RN35	2.00	1.75	1.62
am 17 ssu 1	2.00	1.64	1.47
N21	2.00	1.98	1.84
N22	2.00	1.95	1.93
N25	2.00	1.94	1.86
N31	2.00	1.93	1.84
N32	2.00	1.88	1.74
N35	2.00	1.88	1.85
N36	2.00	1.87	1.74
N41	2.00	1.96	1.96
N42	2.00	1.89	1.76
N43	2.00	1.99	1.93
N45	2.00	1.94	1.88
N46	2.00	1.98	1.94

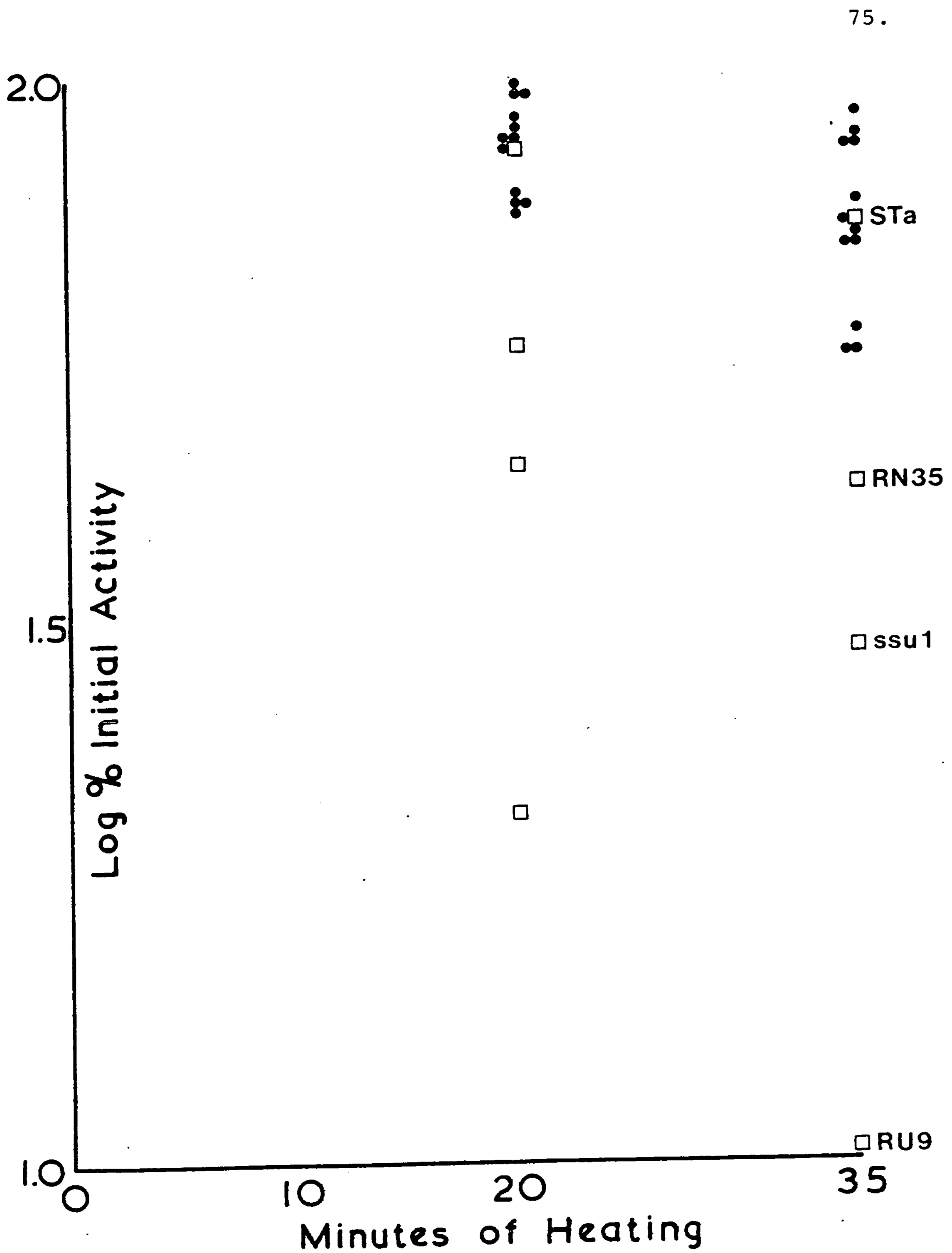


Fig. 2.10 Thermostability Assay Of NA-Induced <u>am 17</u> Revertants At pH8.0 Using Assay System A.

(iv) CONCLUSIONS AND DISCUSSION

One of the main objects of this piece of work was to study the specificity of the mutagen NQO, primarily through the reversion analysis of well-characterised mutants, and to see if as a result anything could be said about the identity of the nonsense codon of the mutant am 17 and the two related nonsense mutants am 109 and am 142.

Rather than study the mutagen NQO in isolation it was considered desirable to include three other mutagens in the study; UV light, nitrous acid and EMS. These mutagens, like NQO, have been well studied in other systems. It is of some interest to know whether these mutagens show the same specificity in Neurospora as they do in other organisms.

In the first part of the study the three well-characterised mutants am 1, am 3 and am 7, were used to test the specificities of the four mutagens.

The mutants am 1 and am 7 both carry the alteration of a GC base pair to an AT base pair. Thus both strains require an AT → GC transition to revert to wild-type. These two mutants are defective in coenzyme binding (Brett et al. 1976) which probably accounts for their inability to revert through secondary changes which lead to intragenic suppression (Stadler, 1966).

The mutant am 3 carries an AT → GC alteration and thus requires a GC → AT transition to revert to wild-type (Brett et al., 1976). This mutant has a defect in the conformation of its GDH and is able to revert through mutations at secondary sites within the gene (Pateman and Fincham, 1965). Thus in order to use this mutant to screen for GC → AT transitions it is necessary to try and identify true wild-types among the revertants through the use of enzyme assays.

These three mutants were used to screen the four mutagens NQO, UV, nitrous acid and EMS for their ability to induce AT → GC and GC → AT transitions.

NQO was found to be able to revert am 1 at low frequencies up to a maximum of 1.8×10^{-6} surviving conidia, but was unable to revert am 7 at anything above 0.1×10^{-6} surviving conidia. However it was found to be able to revert am 3 at much higher frequencies up to 8.6×10^{-6} surviving conidia, of which approximately 30% of revertants (5 out of 18) were indistinguishable from a wild-type strain on the basis of two thermostability assays and no more than 50% deviation from wild-type in the specific activity in assay C. It is not possible to draw conclusions about the relative efficiency of NQO in inducing AT or GC transitions on the basis of reversion studies at two sites within the am gene. This is because considerable variation can exist in the mutability of different sites within a gene. For example the mutants am 1 and am 7 both require the same base change to revert and are only approximately one hundred base pairs apart within the gene (Brett et al., 1976). However NQO is able to revert am 1 at approximately twenty times the frequency at which it can revert am 7. Thus all that these results can tell us is that NQO is able to induce transitions of AT base pairs and probably GC base pairs within the am gene of Neurospora crassa. In all of the major studies of NQO specificity it has been found that there was a marked specificity for GC transitions and transversions. However in most of these studies it was also found that a small proportion of the NQO-induced mutational events involved alterations of AT base pairs (Miller, 1983; Shinoura et al., 1983; Prakash et al.,1974). This is perhaps not too surprising for a mutagen which has been shown to require the operation of error-prone repair systems for the production of its mutagenic effects (Ikenaga et al., 1975; Inoue et al., 1980). Such repair systems are liable to make mistakes at sites which have not been subject to mutagenic attack and hence tend to counteract any apparent mutagen specificity.

UV light was found to be able to revert all three mutants, am 1, am 7 and am 3, at frequencies up to 2.3, 1.0 and 9.6 x 10^{-6} conidia respectively. Approximately 10% (1 out of 11) of the am 3 revertants appear to be true wild-type revertants, and hence represent GC → AT transitions. In prokaryotes UV favours the induction of transitions over transversions with a bias towards GC → AT transitions (Todd and Glickman, 1982; Miller, 1983), while in yeast UV was able to induce all types of transitions and transversions (Sherman and Stewart, 1973). These results show that UV is able to induce both types of transition with comparative ease in Neurospora, and are in agreement with previous studies (Pateman and Fincham, 1965; Seale, 1968).

Nitrous acid proved to be a very poor mutagen in reversion studies with the three mutants am 1, am 3 and am 7. Reversion frequencies with am 1 and am 7 were never higher than 0.2 x 10^{-6} conidia, while am 3 proved to be non-revertible with this mutagen. Nitrous acid is thought to specifically induce both types of transition mutation through the deamination of adenine and cytosine which alters the base pairing specificity of those bases. Such specificity for the induction of both types of transition has been observed in yeast (Stewart et al., 1972; Prakash and Sherman, 1973). Nitrous acid is able to induce AT → GC transitions in this Neurospora system but it is hard to see why it is unable to revert am 3, unless it has a bias towards AT → GC transitions in this system and is such a poor mutagen that GC → AT transitions are induced at undetectable levels. It is also necessary to assume that none of the intragenic suppressor mutations of am 3 arise via AT → GC transitions.

EMS is able to revert am 3 at a frequency of 40 x 10^{-6} conidia, of which approximately 30% (4 out of 14) appears to be wild-type reversion, but is unable to revert either

am 1 or am 7. This is consistent with studies in other systems which show EMS to be a potent mutagen and one which is highly specific for the induction of GC → AT transitions (Prakash and Sherman, 1973; Coulondre and Miller, 1977). This specificity is attributed to the O6-ethyl-guanine alkylation product of EMS which mispairs with thymine to give rise to GC → AT transitions (Lawley et al., 1973).

The next part of the study involved the nonsense mutant am 17. From the work of Seale et al. (1977) it was found that the nonsense codon of am 17 is either amber (UAG) or ochre (UAA). It was thought that if NQO showed a specificity for inducing alterations of GC base pairs in Neurospora, as it has been shown to do in other organisms, then it might be possible to choose between the two alternative nonsense codons.

As is shown in Figure 2.1 an amber codon is able, through single base pair changes, to give rise to seven different amino-acid codons, one of which is the wild-type glutamine codon. An ochre codon is able to mutate to give rise to codons of the same amino acids as amber, except that it gives rise to an opal nonsense codon instead of a tryptophan codon. Of the six possible non-wild-type amino acid replacements only two have been isolated; tyrosine and leucine (Seale et al., 1977). An amber codon gives rise to a tyrosine codon via a transversion of the GC base pair, to a leucine codon via an AT → TA transversion of the middle base pair, and a wild-type glutamine codon via an AT → GC transition of the first base pair. An ochre codon would give rise to leucine and glutamine codons in the same way as an amber codon, but would give rise to a tyrosine codon via a transversion of the third AT base pair.

NQO, UV and NA were all found to revert am 17 at frequencies of up to 45.0, 2.7 and 0.9 x 10^{-6} conidia respectively. EMS was found to be unable to revert am 17. This

is not surprising if EMS is as specific for GC → AT transitions in this system as it is in others, since a GC → AT transition will not convert an amber or an ochre codon to an amino-acid codon.

When the NQO revertants were analysed using various enzyme assays it was found that out of twenty-seven revertants fifteen had tyrosine replacements, nine had mutant tyrosine tRNA supersuppressors, two had leucine replacements, and one had the wild-type glutamine replacement. The nine supersuppressors must have arisen at tyrosine tRNA genes through either an AT → CG or GC → CG transversion if the nonsense codon is amber, or an AT → TA or GC → TA transversion if the codon is ochre.

The results are entirely consistent with NQO being specific for the induction of GC base pair alterations and the nonsense codon being amber (UAG). In this permutation of events the nine supersuppressors would probably have arisen via GC → CG transversions, which would mean that out of the twenty-seven mutational events as many as twenty-four could have resulted from transversions of GC base pairs. This would be in agreement with studies in other systems which suggest that NQO shows a strong tendency to induce alterations of GC base pairs, and would mirror the studies with NQO in yeast which show that NQO is able to revert amber mutants but not ochre mutants (Prakash, 1974).

The alternative explanation is to suppose that the nonsense codon is ochre (UAA) and that NQO is able to induce transitions and transversions of AT base pairs with comparative easeat this site in the gene, with a bias towards AT transversions since at least seventeen of the twenty-seven revertants would have arisen through this alteration. It would also be necessary to assume that lysine, serine and glutamate replacements, which all arise through transversions of AT base pairs, are incompatible with GDH activity

in order to explain their absence from the NQO revertants. As will be seen from the UV and nitrous acid results at least two of these three replacements appear to be possible.

Unfortunately, despite the strong likelihood of the nonsense codon being amber (UAG) and NQO being specific for GC base pair alterations, it is not possible, on the basis of these results, to entirely rule out the possibility that the nonsense codon is ochre (UAA) and that NQO is able to induce AT transversions at relatively high frequencies.

However, subsequent to these studies on am 17 revertants the sequence of the am gene became available (Kinnaird and Fincham, 1983). This revealed that the glutamine codon which gives rise to the am 17 nonsense codon at position 313 in the gene, has the sequence CAG. In the light of this information it is far more likely that the nitrous acid treatment which induced the am 17 nonsense mutant did so via a single GC → AT transition to give rise to an amber (UAG) codon, rather than through simultaneous GC → AT transitions of the first and third positions to give rise to an ochre codon.

Thus one can safely conclude, taking all the evidence into consideration, that am 17 is an amber mutant and that NQO displays the same relative specificity for G·C base pair alterations in Neurospora as it does in other systems. Fifteen out of eighteen identifiable changes represent transversions of a GC base pair. The base pair alterations in the other nine revertants which carry supersuppressor mutations are also likely to represent transversions of GC base pairs. These supersuppressors appear to insert tyrosine which can be coded for by the codons 5'UAC3' or 5'UAU3'. There is strong evidence to suggest that tyrosine tRNA molecules can recognise both codons by virtue of the anticodon sequence 5'GUA3' (Gauss and Sprinzl, 1984). This is because in tRNA-codon interactions guanine can bind with cytosine or uracil. For a tyrosine tRNA molecule to recognise an

amber codon, 5'UAG3', its anticodon must change from 5'GUA3' to 5'CUA3' which represents a GC → CG transversion in the tyrosine tRNA gene. Thus the nine supersuppressor mutants can safely be classified as GC → CG transversions. This means that out of the twenty seven NQO-induced revertants of am 17 twenty four represent transversions of a GC base pair which indicates a high degree of specificity of NQO for inducing alterations of GC base pairs.

Fourteen nitrous acid-induced revertants of am 17 were analysed in the same manner as the NQO-induced revertants. All fourteen revertants appeared to be true wild-type revertants which arise through AT → GC transitions of the first position of the nonsense codon. No supersuppressors, tyrosine or leucine replacements which arise through transversions were observed. This indicates that nitrous acid preferentially induces transitions rather than transversions at this locus which agrees with findings in other systems where nitrous acid was found to specifically induce AT and GC transitions.

The one other amino-acid replacement which could have arisen following an AT → GC transition is the tryptophan replacement which would almost certainly be expected to have an observable effect on enzyme properties. The absence of any nitrous acid induced revertants which seem likely to represent the tryptophan replacement class despite the ability of nitrous acid to induce AT → GC transitions suggests that a tryptophan replacement is incompatible with enzyme activity. This may explain why Seale et al. (1977) were unable to isolate a tryptophan replacement out of twenty-two revertants in an attempt to show am 17 to be an amber mutant.

Despite the strong indication of specificity of these results with nitrous acid it must be pointed out that when Seale (1968) analysed twenty-five nitrous acid induced revertants of am 17 he was able to characterise only fifteen

as wild-type. The other ten revertants, with hindsight, appear to be made up of three tyrosine replacements and seven supersuppressed revertants. These results suggested that nitrous acid showed a preference for AT → GC transitions but not to the same extent as the findings of this study would indicate.

The eleven UV induced revertants of am 17 which were analysed appeared to fall into five classes. The largest class with six representatives appeared to consist of true wild-types. There were also two apparent leucine replacements and a tyrosine replacement. The remaining two revertants appeared to represent novel classes of revertant with unidentifiable replacements.

The likelihood that six of the eleven revertants arose via AT → GC transitions suggests that UV shows a certain degree of specificity towards transition mutations in this system, but it is also clear from the occurrence of other revertant classes that UV is able to induce transversions at relatively high frequencies also. This agrees with findings in yeast where UV was able to induce all six types of transition and transversion (Sherman and Stewart, 1973), and in E. coli where the same mutation spectrum was observed but with a marked specificity for transitions (Miller, 1983; Todd and Glickman, 1982).

In previous studies on UV revertants of am 17, Stadler (1966) found that ten out of fourteen revertants were wild-type and hence observed UV-induced specificity for transitions at this locus. Of the other four revertants three appeared to be leucine replacements and one appeared to represent a novel class of revertant. This novel revertant resembled neither of the two novel revertants isolated in this study, having wild-type heat stability at pH 8.0 but approximately 10% of wild-type stability at pH 6.5.

It is not worthwhile speculating about which novel class of revertant may represent which amino-acid replacement.

There are four possible non-wild-type replacements apart from leucine and tyrosine, and these are lysine, glutamic acid, serine and tryptophan. In the discussion of the nitrous acid induced am 17 revertants it was pointed out that the ability of nitrous acid to induce AT → GC transitions should facilitate the induction of tryptophan replacements. The absence of these replacements from this and Seale's study (1968) on nitrous acid revertants suggests that tryptophan is incompatible with enzyme activity. This would leave lysine, glutamate and serine as the likely candidates for the two novel UV induced revertant classes of this study, and the unidentified revertant class of Stadler's study (1966).

CHAPTER III: FRAMESHIFT MUTAGENESIS

(i) INTRODUCTION

Frameshift mutations involve the loss or gain of one or more base pairs in the coding sequence of a gene. The earliest model of frameshift mutagenesis arose from the work of Streisinger et al. (1966) who inferred the exact DNA changes in T4 lysozyme frameshift revertants from amino-acid sequence data. Their model suggested that the presence of repeated sequences in the DNA (e.g. monotonous runs of doublets) could allow strand slippage and misalignment during replication and repair. This would result in bases in the template strand being either excluded from or included twice in the newly synthesised copy. The model predicts that deletions and insertions will subtract from or add to runs of identical bases or short sequences.

Early work with proflavin induced frameshifts in the T4 lysozyme gene showed that out of twelve mutants all but one could be accounted for by the Streisinger model (Roth, 1974). The exception could be explained with a minor modification of the basic scheme. Interestingly, of the twelve mutants ten were additions, an apparent bias that remains unexplained.

Subsequent studies in E. coli and Salmonella typhimurium using ICR compounds (acridines with alkylating side-chains) also supported the Streisinger model (Roth, 1974; Isono and Yourno, 1974); frameshifts tended to occur in runs of GC base pairs. The apparent specificity of ICR compounds for GC base pairs is thought to be due to the preferential reaction of the alkylating side-chain with the N-7 position of guanine.

Much work has been done on frameshift mutagenesis in yeast by Stewart and Sherman (1974). They looked at frameshift mutations in the first forty-four bases of the coding sequence of the gene for iso-1-cytochrome C, using amino acid sequence analysis to identify the changes. The reverting frameshifts were often, though not always, found to occur at sites consistent with the Streisinger model.

Thirteen percent of the mutations studied (13 out of 97) were found to represent more complex events, involving deletions or duplications accompanied by adjacent base pair substitutions. These could not be accounted for by the Streisinger model.

A wide range of mutagens was used in this study, but ICR-170, which is generally an effective frameshift inducer, was not found to induce frameshifts in the region of the gene under investigation. This was despite the presence of the sequence GGCCGG which, in *Salmonella*, had been found to be susceptible to ICR-170.

In an attempt to explain the thirteen percent of yeast frameshift mutants which were not accounted for by the Streisinger model Ripley (1982) put forward a new model based on quasi-palindromic DNA sequences. She suggested that such sequences could form imperfect hair-pin loops during DNA synthesis, and that these might be processed, either by repair or by misreplication, to give both frameshifts and base pair substitutions. One of the yeast double frameshifts, which occurred six times, could be explained precisely by Ripley's model, as could complicated frameshift mutations in T4 bacteriophage (Ripley and Glickman, 1984).

The most effective frameshift mutagens are those that possess a heterocyclic ring structure and hence the ability to stack with DNA bases. According to the Streisinger model such compounds would facilitate frameshift mutations by stabilising misaligned base pairing. The ability to intercalate between base pairs may also be important. Lee and Tinoco (1978), using NMR, showed that a mixture of the oligonucleotides G_pU_pG and C_pC produces, in the presence of ethidium bromide, a minihelix containing two G·C base pairs with the U forming an external bulge, and with the ethidium intercalated between the two base pairs.

Frameshift mutations can be induced by mutagenic agents such as UV, nitrous acid and alkylating agents

(Sherman and Stewart, 1973; Miller, 1983). While these agents are not able to stabilise Streisinger model intermediates it is thought that they are able to create the conditions under which such base-slippage intermediates can form, through the induction of repair processes, and hence facilitate frameshift mutagenesis. It is also thought that certain mutagens can induce frameshifts through the creation of apurinic or apyrimidinic gaps in the DNA. Such gaps are known to be mutagenic in E. coli (Schaaper et al., 1982). Glycosylase enzymes which specifically release damaged bases from DNA, leaving gaps, have been identified in E. coli and mammalian cells (Lindahl, 1982) and are thought likely to exist in most organisms. Certain chemical modifications of bases are also known to destabilize base-sugar bonds and lead to spontaneous loss of bases. Under certain conditions it is possible that single base gaps in the DNA may remain unrepaired and hence lead specifically to single base deletions. Such specificity has been observed for UV-induced frameshift mutagenesis in some systems (Miller, 1983).

Two am mutants were used to carry out a limited study of frameshift specificity in Neurospora. The mutant am 6 has been shown to have an insertion of a cytosine in the fifth codon of the gene (Siddig et al., 1980). The am 6 mutant was characterised through amino-acid sequencing of revertants, induced with UV, which had secondary compensating frameshift mutations. These studies revealed that UV light is able to induce a variety of frameshift alterations ranging from single base deletions to more complex insertion-deletion events.

A second mutant, am 15, had been characterised, prior to these studies, as a frameshift mutant on the basis of its non-complementation with other mutants and its high reversion rate with the frameshift mutagen ICR-170. UV light was unable to revert am 15 (Seale, 1968).

These two mutants were used in reversion tests to screen the ability of the mutagens ICR-170, UV light, NQO and EMS to induce frameshift mutations. In order to supplement these studies it was considered necessary to characterise the *am 15* mutant by cloning the mutant *am* gene into a bacteriophage vector and determining its nucleotide sequence. The strategy used for the cloning and sequencing of the *am 15* mutant was the same as that used for the cloning (Kinnaird *et al*., 1982) and sequencing (Kinnaird and Fincham, 1983) of the wild-type *am* gene.

In addition, two of the revertants of *am 15*, isolated after treatment with the mutagen ICR-170, and selected as representatives of two distinguishable classes, were also cloned and sequenced. It was thought that these sequences might provide some clues as to the mechanisms by which a frameshift mutagen induces frameshift mutations in *Neurospora*.

(ii) MATERIALS AND METHODS

(1) ANALYSIS OF REVERTANTS

All of the media, stock maintenance, reversion analysis and enzyme assays used in these studies are as described in Chapter II.

(a) Mutagen: the treatment procedures concerning UV, NQO and EMS are as described in Chapter II. ICR-170, 6-Chloro-9-(3-N-(2-chloroethyl)-ethylamino)-propylamino-2-methoxyacridine dihydrochloride, was obtained from Terochem Laboratories Ltd., Canada. For treatment of conidia it was dissolved in distilled water before adding to the conidia suspension. Treatment was at 30°C for 2 hours.

(b) <u>Non-denaturing poly-acrylamide gel electrophoresis (P.A.G.E.) (Laemmli, 1970)</u>

Solution A:	Tris	36.6g
	1M HCl	48 mls
	T.E.M.E.D.	0.46 mls
	H_2O	to 100 mls

pH to 8.9 and filter solution.

Solution C:	Acrylamide	26 g (gives 6.5% gel)
	Bis-acrylamide	0.693g
	$K_3 Fe (CN)_6$	15mg
	H_2O	to 100 mls

filter solution.

For 100 mls of gel use 25 mls solution A, 25 mls of solution C and 50 mls H_2O. To polymerise gel add 1 ml of 0.14g ml^{-1} $(NH_4)_2 S_2 O_8$.

The gel for analysis of revertants was set up in a 16 cm gel apparatus (e.g. Bio-Rad Protean) using 1.5 mm spacers and a plastic comb. Samples of crude extract were loaded with a syringe and needle, after being mixed with bromophenol blue marker and sucrose to weigh down the sample.

The tank buffer (pH 8.3) consisted of 0.6 g Tris and 2.88 g glycine in 1 l of H_2O. Samples were run at 50mA until marker dye had travelled required distance.

GDH Specific Stain:

Sodium Glutamate	0.3g
NADP	10mg
PMS (added last)	10mg
NBT	10mg
0.1M Tris HCl pH 8.5	40mls

The gel was stained for 5 minutes at 35°C or until GDH activity showed up as bands in the gel. The gel was stored in 7% acetic acid.

(2) CLONING AND SEQUENCING

(a) Bacterial Media

LB medium:	Oxoid Tryptone	10g l^{-1}
	Difco yeast extract	5g l^{-1}
	Na Cl	10g l^{-1}

BBL medium:	Baltimore Biological Laboratories Trypticase	10g l^{-1}
	Na Cl	5g l^{-1}

Solid media contained either 15g l^{-1} agar (bottom)

or 7g l^{-1} agar (top)

2TY medium:	Oxoid tryptone	16g l^{-1}
	Difco yeast extract	10g l^{-1}
	Na Cl	5g l^{-1}

(b) Bacterial Strains: (all E. coli)

Q358:hsd R_k^-, hsd M_k^+, sup F, Ø80^r (Karn et al., 1980)

Q359:hsd R_k^-, hsd M_k^+, sup F, Ø80, P2 (Karn et al.,1980)

JM101: lac$^-$, pro$^-$, sup E, thi$^-$, F$^-$tra D36, pro AB, lac I^q, ZΔM15 (Messing et al., 1977).

Bacteriophage Strains

λL47: Size - 40.6kb

Genetic Markers - (srIλ1-2)$^{\nabla}$, imm434 cI^-, NIN5, chi A131

Restriction insertion sites - EcoRI, Hind III, Bam HI (Loenen and Brammar, 1980).

M13 mp8: (Messing and Vieira, 1982).

(c) Preparation of *Neurospora* DNA

Neurospora mycelium was grown up in 500mls Vogel's liquid minimal medium (plus glutamate if required) in a 2 l flask at 30°C for about 24 hours. A heavy inoculum of conidia was used. The mycelium was filtered and then freeze-dried. 2g was generally sufficient for a yield of 1 - 2mg of DNA.

DNA was prepared using a method similar to that of Cryer *et al*. (1975).

(d) Restriction digests

The four restriction enzymes used in this study were Hind III, Bam HI, Xho I and Sal I (Boeringer and BRL).

Hind III and Bam HI require an incubation buffer made up of 50mM Tris-HCl (pH 7.5), 50mM Na Cl, 10mM Mg Cl_2, and 1mM β-mercaptoethanol.

Xho I and Sal I require a higher salt incubation buffer with 100mM Na Cl.

The incubation buffers were made up as 10 x stocks.

Restriction digests were carried out at 37°C for one hour using two units of enzyme per μg of DNA.

If the DNA was run on an agarose gel then a stop-solution was added to 20% total volume consisting of 100mM EDTA, 20% Ficoll 400, 0.05% bromophenol blue (pH 7.5). The bromophenol blue is a marker dye for following the progress of the electrophoresis.

If λDNA is present in the digest then heating at 70°C for 10mins followed by rapid cooling denatures the λ cohesive ends before running on a gel.

If a digest was to be used in a ligation reaction, the restriction enzymes were inactivated by heating at 70°C for 10 minutes.

(e) Agarose gel electrophoresis

Two types of agarose gel were used, a flat-bed gel, and a mini-gel.

The flat-bed gel was run with a tris-acetate buffer:

10 x tris-acetate - 0.4M Tris base
0.2M Sodium acetate trihydrate
0.02M EDTA
0.18M Na Cl

pH to 8.2 with acetic acid and filter.

To make the gel, 150ml of 0.7% agarose was made up with tris-acetate buffer and boiled until the agarose was dissolved. The gel was then poured with a comb in place. When set the comb was removed and the DNA samples loaded. The gel was run at no more than 5V cm^{-1}.

Mini-gels were run with a tris-phosphate buffer:

10 x tris-phosphate - 0.9M Tris base
15.5ml l^{-1} 85% phosphoric acid
0.02M EDTA

pH to 8.3 and filter.

To make a mini-gel, 50ml of 0.7% agarose was made up with Tris-phosphate buffer and boiled until dissolved. Tape was wrapped round an 8cm^2 glass plate to form a tray for pouring the gel into.

The gel was run at up to 10V cm^{-1}.

The flat-bed gels were stained in 500ml of Tris-acetate buffer plus 50μl 10mg ml^{-1} ethidium bromide, and the mini-gels in 50mls. Tris-phosphate buffer plus 5μl 10mg ml^{-1} ethidum bromide, for approximately 30 minutes.

The DNA in the gels could then be visualised on a UV light box and photographed if necessary.

(f) Ligation

T4 DNA ligase was used with a ligase buffer made up as a 10 x stock: 0.66M tris-HCl (pH 7.6), 10mM EDTA (pH 7.0), 0.1M Mg Cl_2, 0.4M Na Cl. Reactions were carried out in the presence of 5mM DTT and 1mM ATP (pH 7.0) at 12°C overnight.

(g) Packaging of λDNA molecules

After Neurospora DNA fragments had been ligated into the λL47 vector it was necessary to package the λDNA with previously prepared coat proteins to make viable recombinant phage particles. This in vitro packaging was carried out according to Grosveld et al. (1981).

(h) Infection of E. coli Q358 and Q359 with λ

Q358 is a permissive host which allows recombinant and non-recombinant λ growth, while Q359 is a restrictive host which supports only recombinant λ growth.

10ml LB were inoculated from a single colony and incubated overnight at 37°C.

20ml LB were inoculated with 0.5ml overnight culture and shaken in an orbital incubator at 240 rev min^{-1} and at 37°C to an OD of about 0.5 at 600nm.

The bacteria were spun down at 7 krpm for 10min and resuspended in 20ml of 10mM Mg SO_4.

100μl of an appropriate dilution of phage was added to 0.5ml bacteria and incubated at 37°C for 20min.

3ml of molten (45°C) BBL top agar, containing 10mM Mg SO_4, was added with swirling and poured as a top layer on a BBL bottom agar plate.

Plates were incubated upside-down at 37°C overnight.

(i) In situ hybridisation

Phage were transferred as plaques to nitrocellulose by the method of Benton and Davis (1977).

Double-stranded DNA was labelled by nick translation following the procedure of Maniatis *et al*. (1982). Unincorporated ^{32}P-dCTP was removed from labelled probe by the spun column procedure of Maniatis *et al*. (1982).

Pre-hybridisation of filters was carried out in sealed plastic bags with 10 x Denhardt solution, 4 x SSC and 50μg ml^{-1} denatured salmon sperm DNA for 1 hour at 65°C.

Hybridisation was also performed in bags with 6 x SSC, 50μg ml^{-1} denatured salmon sperm DNA, 0.1% SDS and labelled probe DNA (at least 10^6cpm) overnight at 65°C.

The filters were washed in 4 x (plus 0.1% SDS), 2 x, 1 x and 0.1 x SSC for 1 hour, 30 min, 30 min and 15 min respectively at 65°C.

After baking dry between paper at 80°C for 20 min in a vacuum oven, the filters were marked with radioactive ink and laid down with Kodak Ortho-G X-ray film in cassettes.

(j) Preparation of λ clone DNA

DNA was prepared from λ clones of the mutant *am* genes by the procedure of Kinnaird *et al*. (1982).

(k) Recovery of DNA from agarose gels

DNA was eluted from gels onto strips of NA-45 DEAE membrane (Schleicher and Schnell) according to the procedure of Dretzen *et al*. (1981).

(l) Transfection of *E. coli* JM101 with M13 mp8

To make competent JM101 cells 20ml of LB was inoculated with 0.5ml of an overnight culture of JM101 and shaken at 240 rev min^{-1} at 37°C to an OD of 0.3 at 600nm.

The bacteria were spun down at 7 krpm for 10 min, resuspended in 10ml ice-cold 50mM Ca Cl_2, and left for 30 min on ice.

The cells were spun down once more and resuspended in 2ml ice-cold 50mM Ca Cl_2.

300μl of competent cells were incubated with no more than 5ng M13 DNA on ice for 30 min.

The cells were then heat shocked at 42°C for 5 min.

200μl of log phase JM101 cells, 25μl of 2% X gal (in DMF), 25μl of 2.5% IPTG (in H_2O) and 3ml LB top agar (45°C) were then added to the transfection tubes, swirled to mix, and top plated onto LB bottom agar plates.

Plates were incubated upside down at 37°C.

Non-recombinant M13 plaques are stained blue on this medium, while recombinant M13 plaques are colourless.

(m) <u>M13 DNA mini-preparation</u>

A plaque was toothpicked into 1.5ml 2TY medium with 15μl of an overnight culture of JM101 and shaken at 240 rev min^{-1} at 37°C for 4½ to 5½ hours.

The bacteria were spun out in Eppendorf tubes for 5 min and 1ml of supernatant was mixed with 250μl 20% P.E.G., 2.5M Na Cl.

P.E.G. precipitated phage were spun out for 5 min and resuspended in 100μl TE.

Protein coats were extracted with Tris-equilibrated phenol, the phenol was extracted with ether, and the DNA precipitated with 250μl -20°C 95% ethanol.

The DNA was taken up in 50μl TE and stored at -20°C.

(n) <u>Annealing of M13 clones</u>

M13 single-stranded DNA clones were annealed in annealing buffer (10mM Tris pH 7.4, 10mM Mg Cl_2, 50mM Na Cl) at 65°C for 1 hour in a volume of no less than 20μl, and run on agarose gels.

After staining with ethidium bromide the double-stranded DNA formed from annealed complementary strands was seen to have migrated more slowly than the un-annealed single-stranded DNA.

(o) Sequencing of clones

Sequencing of the clones was performed by the dideoxy chain termination method of Sanger *et al*. (1980) using a synthetic 17-base M13-specific primer.

(iii) RESULTS OF REVERSION ANALYSIS

(a) Reversion Analysis of am 6 and am 15 with the Mutagens ICR-170, UV, NQO and EMS.

(1) ICR-170

Table 3.1 gives the results of the treatment of am 6 and am 15 with the mutagen ICR-170.

The well-characterised mutant am 6, which has an insertion of a cytosine in the fifth codon of the gene, was not found to revert after ICR-170 treatment.

Not surprisingly, the am 15 mutant, which was classified as a frameshift mutant on the basis of its revertibility with ICR-170, is able to revert at relatively high frequencies with ICR-170 in these studies.

(2) UV

Table 3.2 gives the response of the two mutants to UV treatment.

It was already known that am 6 is able to revert after UV treatment and this is confirmed in these studies. However the am 15 mutant appears to be refractory to UV-induced reversion and only reverts at frequencies of 0.2×10^{-6} surviving conidia.

Interestingly the response of the two mutants to UV treatment is the opposite of their response to ICR-170 treatment with am 6 reverting with the former but not the latter and am 15 hardly reverting with the former but reverting with the latter.

(3) NQO

The results of the NQO treatment of both mutants are given in Table 3.3.

NQO appears to be able to revert both mutants but at very low frequencies. This suggests that NQO is a poor frameshift inducing mutagen.

TABLE 3.1. ICR-170 Reversion Analysis of am 6 and am 15

	Dose/µg ml^{-1}	% Survival	Revertants	Reversion Freq./10^{-6}
am 6:	0	100	0	0
	2	86	0	0
	4	65	0	0
	6	43	1	<0.1
am 15:	0	100	0	0
	2.5	59	114	9.2
	5.0	11	74	26.4
	7.5	3	22	27.5

TABLE 3.2. UV Reversion Analysis of am 6 and am 15

	Dose/secs	Survival	Revertants	Reversion Freq./10^{-6}
am 6:	0	100	0	0
	15	81	43	3.6
	30	35	88	16.6
	45	10	38	25.3
am 15:	0	100	0	0
	20	68	0	0
	40	45	2	0.2
	65	21	2	0.2

TABLE 3.3. NQO Reversion Analysis of am 6 and am 15

	Dose/μg ml^{-1}	Survival	Revertants	Reversion Freq./10^{-6}
am 6:	0	100	0	0
	0.25	95	2	0.1
	0.5	73	3	0.2
	1.0	38	6	0.7
am 15:	0	100	0	0
	0.25	75	1	<0.1
	0.5	39	3	0.4
	1.0	11	0	0

TABLE 3.4. EMS Reversion Analysis of am 6 and am 15

	Dose %	Survival	Revertants	Reversion Freq./10^{-6}
am 6:	0	100	1	0.1
	1	78	14	1.9
	2	49	20	4.0
	5	17	9	5.1
am 15:	0	100	0	0
	1	95	1	<0.1
	2	83	0	0
	5	18	0	0

(4) EMS

The two mutants show a similar response with EMS treatment as with UV. am 6 responds well to EMS treatment, being able to revert with a frequency of 5.1×10^{-6} surviving conidia, while am 15 produced only one detectable revertant after EMS treatment (see Table 3.4).

(b) Analysis of ICR-170 Induced Revertants of am 15

From the studies which were carried out by Siddig et al. (1980) on the UV-induced revertants of am 6 it was found that UV was able to induce a wide spectrum of compensating alterations and at least seven different classes of revertant were isolated.

Sixteen ICR-induced revertants of am 15 were isolated and analysed using thermostability assays, specific activity measurements, and polyacrylamide gel electrophoresis in an attempt to determine the range of revertants induced by ICR-170.

(1) Enzyme Analysis

A thermostability assay was carried out on crude protein extracts of the sixteen revertants prepared at pH 8.5 and heated at 60°C before being assayed with system C. In addition the specific activity of the extracts in assay C was also calculated from protein estimations. The results are presented in Table 3.5.

Four out of the sixteen revertants showed roughly wild-type thermostability while the other twelve revertants were completely inactivated after five minutes at 60°C. All the revertants showed less than wild-type specific activity in assay system C ranging from 57% to 83% of wild-type.

(2) Polyacrylamide Gel Electrophoresis (P.A.G.E.)

In a previous study several ICR-induced revertants of am 15 were analysed by P.A.G.E. (Fincham, personal communication). The GDH from these revertants was found to

TABLE 3.5. GDH Thermostability and Specific Activity of ICR-Induced am 15 Revertants

GDH activity was assayed in system C at pH 8.5 after heating at 60°C for 0, 15 and 30 minutes.

Strain	% Initial Activity			% Wild-Type
	0 Mins	15 Mins	30 Mins	Specific Activity
STa	100	42	25	100
R7	100	35	22	62
R12	100	34	14	83
R14	100	32	17	75
R18	100	37	18	78

Another 12 Revertants were Inactive after 5 Minutes at 60°C but Showed Zero Time Specific Activities of Between 57% and 75% Wild-Type.

have reduced electrophoretic mobility in comparison with wild-type GDH, and to be too unstable for purification.

When crude protein extracts from the sixteen revertants isolated in this study were run on polyacrylamide gels and the gels stained for GDH activity it was found that the revertants all had altered mobility and fell into three groups (see Fig. 3.1).

The first group (tracks 3, 5 and 10) gave bands which had a comparable staining activity to wild-type but had about 93% of wild-type mobility. This group corresponded to those four revertants which displayed roughly wild-type stability to heat inactivation in the enzyme assays.

The second group (tracks 7 and 9) also had about 93% of wild-type mobility on the gel but gave a less strongly staining band. This group included ten of the revertants which had been inactivated by five minutes at 60°C in the thermostability assay.

The third group (tracks 4 and 8) gave extremely weakly staining bands on the gel and had only about 82% of the electrophoretic mobility of wild-type GDH. This group is represented by two of the twelve thermally unstable revertants and is indistinguishable from the other ten revertants on the basis of zero-time specific activity (Table 3.5).

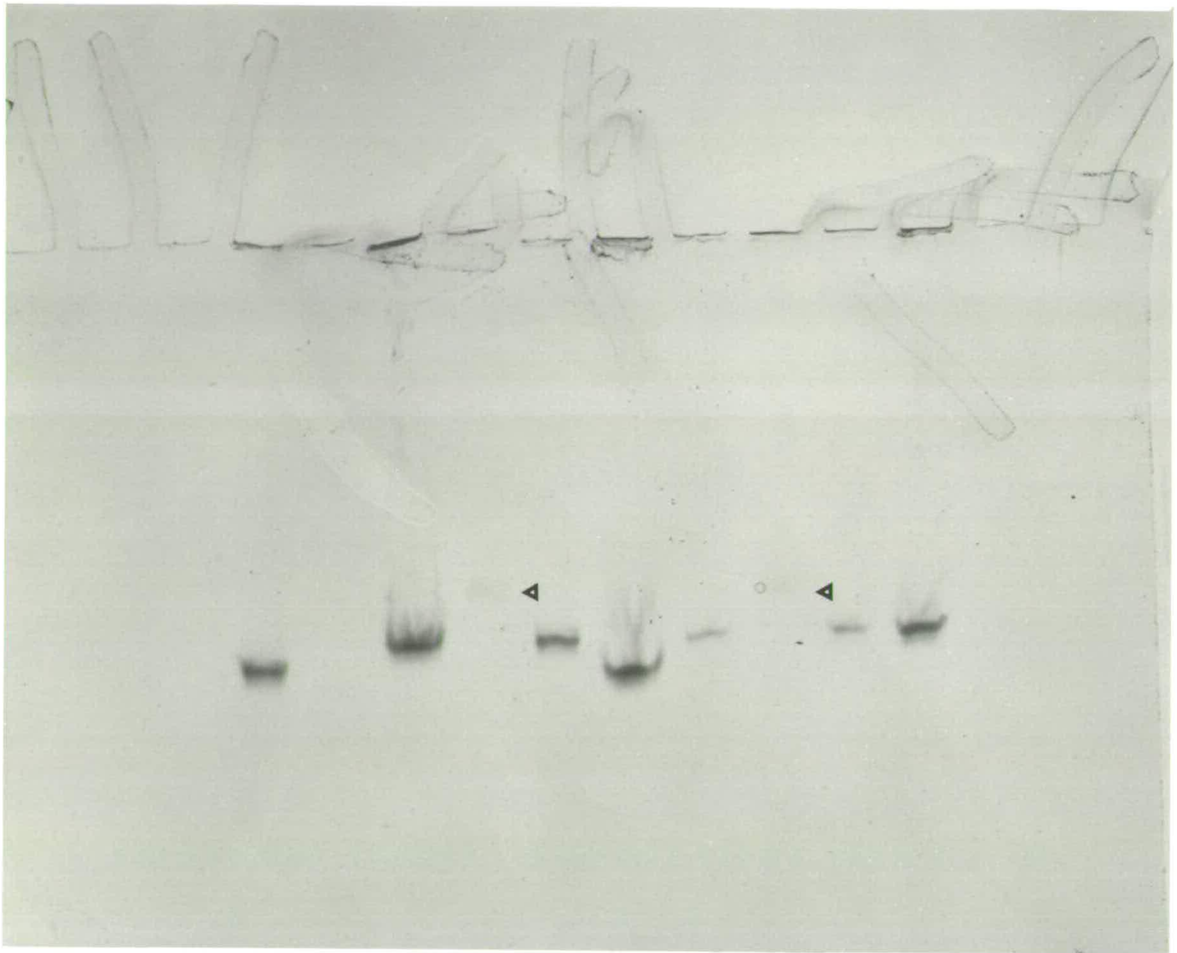


FIGURE 3.1. Non-denaturing P.A.G.E. of ICR-170-induced <u>am 15</u> revertants.

(iv) CLONING AND SEQUENCING OF am 15 AND TWO ICR-INDUCED REVERTANTS, R12 AND R15.

(a) Preparation of DNA for Cloning.

DNA was prepared from the three strains and its optical density measured to check its purity.

As a further test of purity the DNA was digested with Hind III, Bam HI and Eco RI. Figure 3.2 shows that the undigested DNA was of relatively high molecular weight and that it was digested efficiently by the enzymes to give a range of fragment sizes in each case.

The gel in Figure 3.2 also had a tract with Hind III-digested wild-type DNA. The DNA from this gel was denatured *in situ* and transferred to a nitrocellulose filter by the method of Southern (1975). The filter was then hybridised with a nick-translated λ clone of the wild-type *am* gene. The autoradiograph from the hybridised filter confirmed that the *am* gene in the *am 15* strain is contained within the same 9 kb Hind III fragment as it is in the wild-type strain.

(b) Construction of genomic library

Genomic libraries of the three strains were made by ligating 1μg of Hind III digested Neurospora DNA into the Hind III site of the cloning vector λL47. This phage will not grow in a P2 lysogen (such as *E. coli* Q359) unless the central genomic region, containing *red* and *gam*, is replaced by other DNA. The ligated DNA was packaged *in vitro* and titred for numbers of recombinant and viable phage particles on the restrictive and permissive host *E. coli* strains Q359 and Q358 respectively.

Good packaging efficiencies were achieved of between 10^6 and 10^7 p.f.u.s. per μg of λDNA. The proportion of recombinants was low (under 5% for all three *am*$^-$ strains) but this still provided large enough numbers of recombinant phages for clone selection.

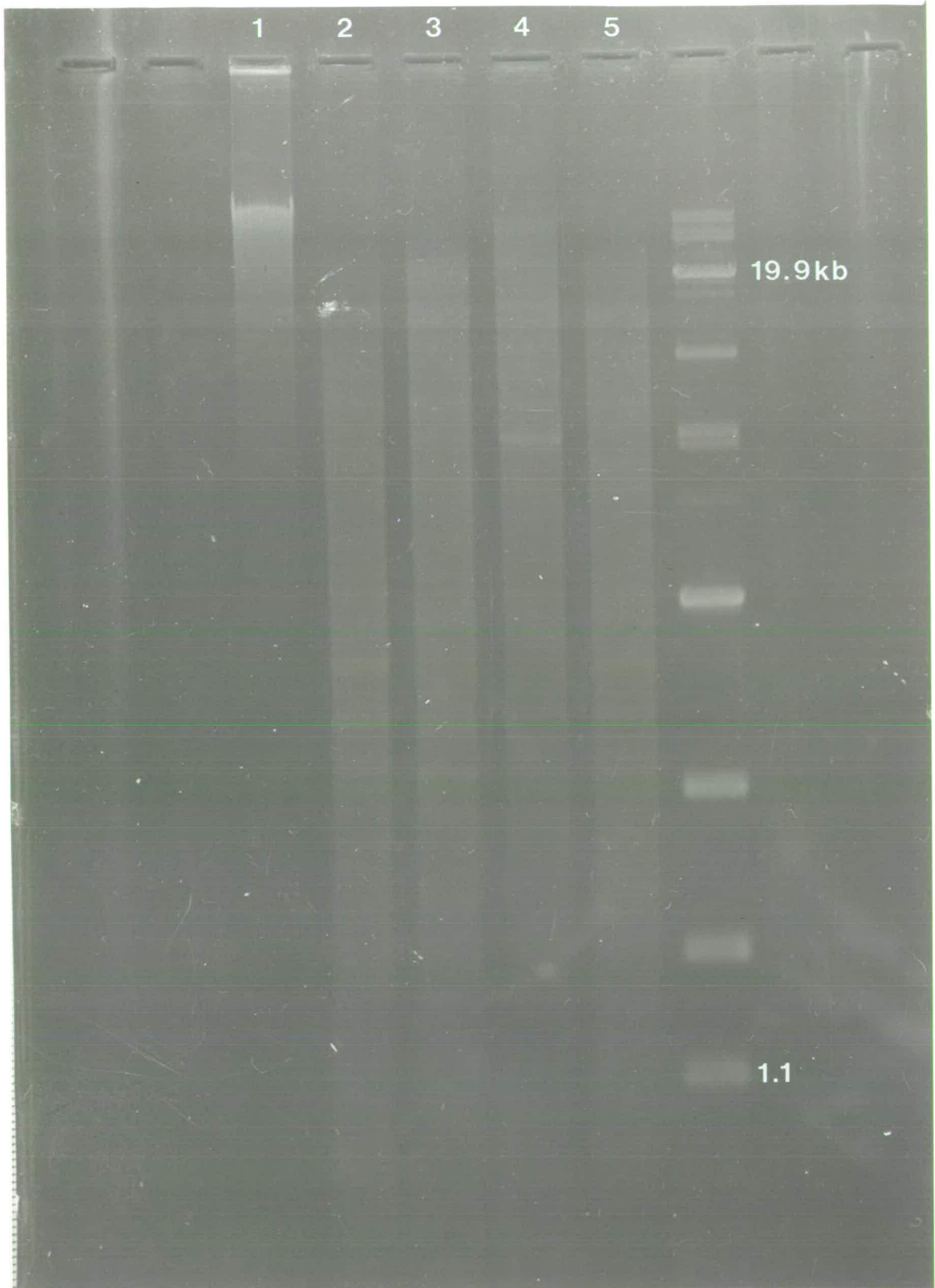


FIGURE 3.2. Restriction digest of am 15 DNA with 1) no enzyme 2) Hind III 3) Eco RI 4) Bam HI, and wild-type DNA with 5) Hind III.

(c) Clone Selection

Enough packaged phage to give between 5 and 10 x 10^3 recombinant plaques were plated on the restrictive host Q359. The phage were then harvested from the plates and replated on the permissive host Q358 at a density of around 10^4 per 14cm plate. The Q358 strain gives bigger plaques which produce better signals for in situ hybridisation.

The almost confluent plaques were then transferred to nitrocellulose filters using the Benton and Davis transfer technique (1977). After denaturing the phage DNA the filters were subjected to in situ hybridisation with a nick-translated Bam HI fragment containing the wild-type am gene. Any plaques which gave positive signals after autoradiography were picked and purified by replating at a lower density (e.g. 100 per 9cm plate) and screening again (see Fig. 3.3).

(d) Preparation of Clone DNA

Once the desired clones had been obtained it was necessary to sub-clone them into the sequencing vector M13 mp8. DNA was prepared from the λL47 clones by the procedure of Maniatis (1978). The DNA was digested and run on an agarose gel to compare it with the L47 clone of the wild-type gene (see Fig. 3.4).

(e) Sub-cloning into M13 mp8 Sequencing Vector

It was known from mapping studies that the mutation am 15 was close to the 5' end of the gene. The 2.7 kb Bam HI fragment containing the gene has five Xho I sites and so generates four Xho I fragments (see Fig. 3.5). The Xho I fragment closest to the 5' end of the gene was thought most likely to contain the am 15 site.

In order to clone the appropriate Xho I fragment into the M13 sequencing vector it was first necessary to isolate

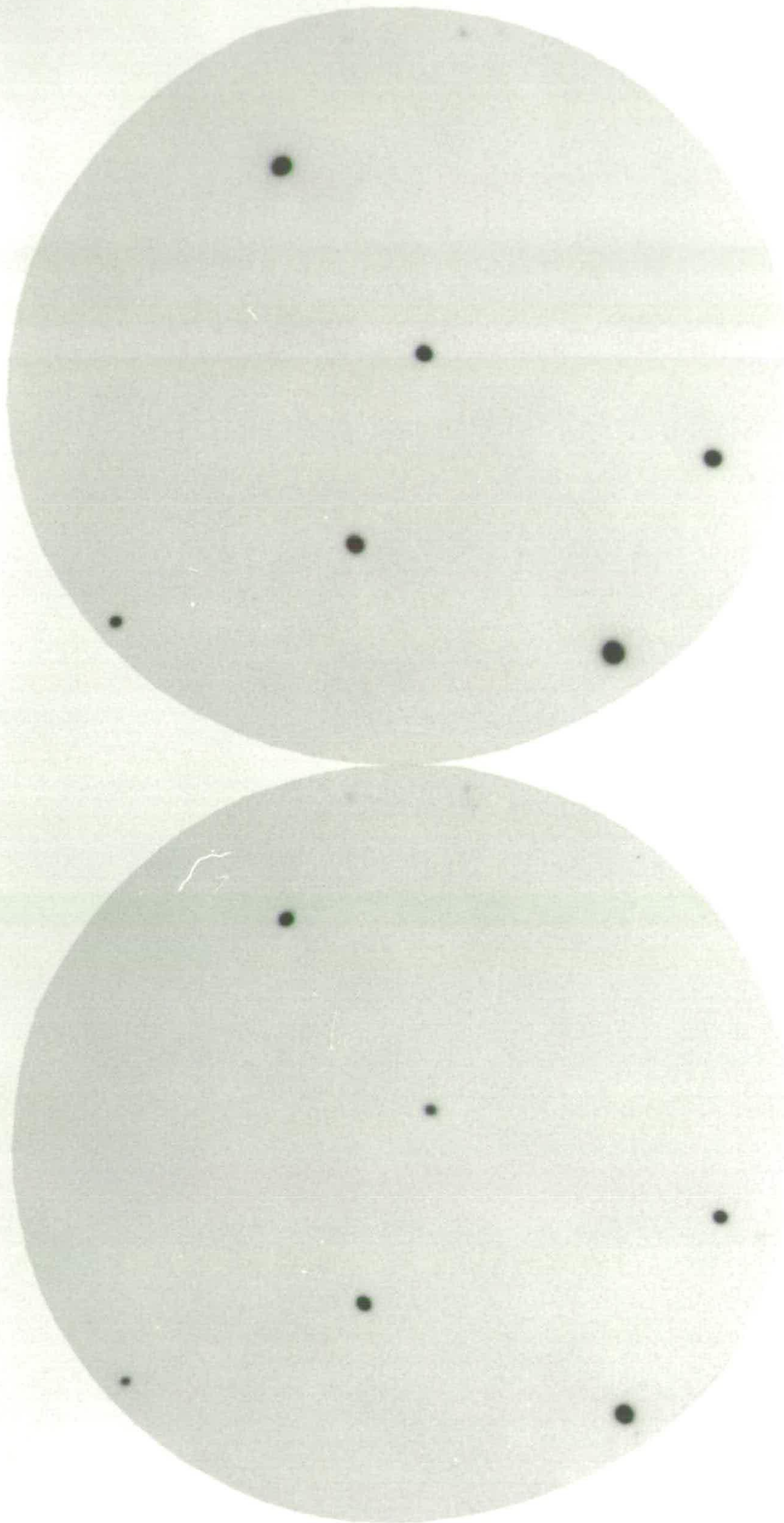


FIGURE 3.3. Six positive signals obtained after clone selection from a λ library of am 15 R15 Hind III-digested DNA.

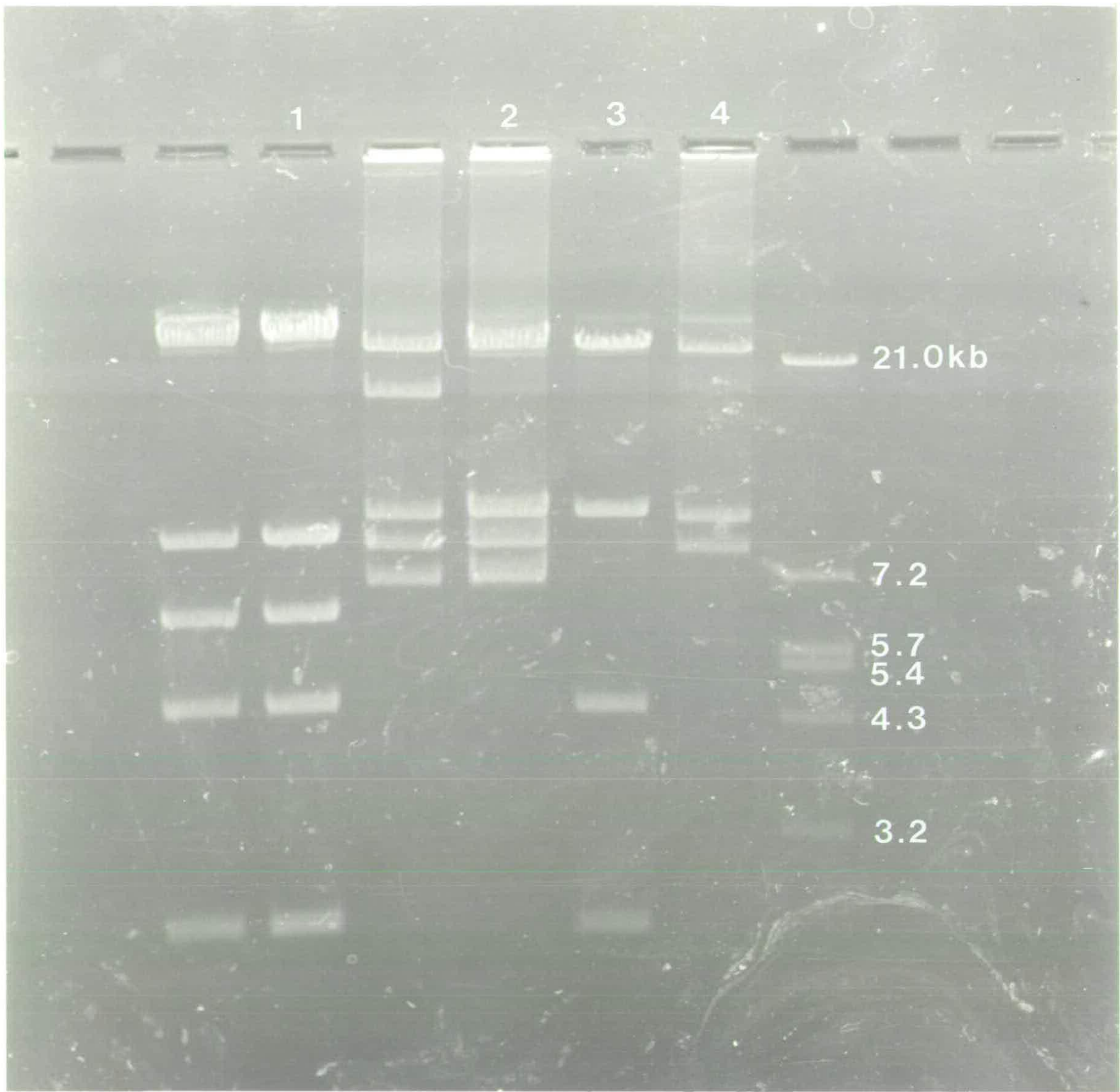


FIGURE 3.4. Restriction digest of am 15/λ clone with 1) Bam HI and 2) Hind III, and of am^{+}/λ clone with 3) Bam HI and 4) Hind III.

Resulting fragment sizes were

1) 23.6, 8.4, 6.5, 4.7 and 2.7kb

2) 23.1, 9.0, 8.2 and 7.2kb

3) 23.6, 10.4, 4.7 and 2.7kb

4) 23.1, 9.0 and 8.2kb

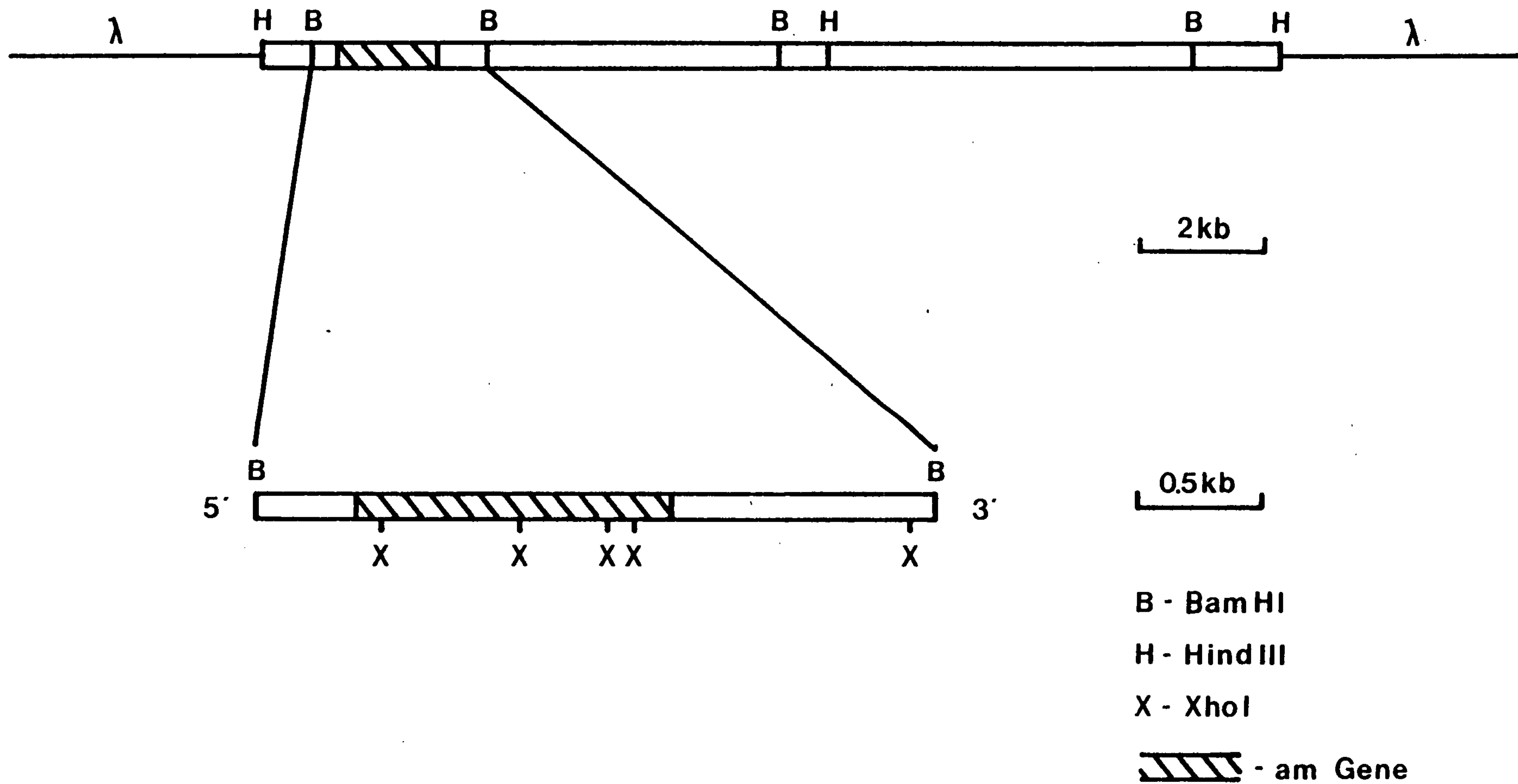


FIGURE 3.5. Restriction site map of the am 15/λ clone.

the 2.7 kb Bam HI fragment from the L47 clones. This was done by running a large Bam HI digest of the λL47 clone DNA (10μg) on a wide track and eluting the 2.7 kb band from the gel by the method of Dretzen *et al.* (1981).

The 2.7 kb fragment DNA was then digested with Xho I and ligated into the Sal I site of M13 mp8. Recombinants were selected by transfecting *E. coli* JM101 and plating out on Xgal medium. The cloning site in the M13 vector is within a *lac Z* gene. M13 phage without inserts in this gene produce plaques which are stained blue on Xgal medium while phage with inserts give rise to clear plaques.

M13 is a single-stranded DNA bacteriophage. Thus M13 clones produced upon infection of JM101 are in single-stranded form. It follows therefore that when four Xho I fragments are ligated into double stranded M13 mp8 DNA eight different single-stranded clones are produced upon infection of JM101.

J. Kinnaird kindly made available the M13 wild-type *am* Xho I clone which was complementary to the M13 mutant *am* Xho I clone required for sequencing of the 5' end of the mutant *am* gene. DNA preparations were made from a number of M13 mutant *am* clones and from the M13 wild-type clone and annealing reactions were set up to identify the required clone. Figure 3.6 shows the DNA run on an agarose gel after annealing reactions had been carried out. Two of the clones clearly migrate more slowly than the others as a result of annealing with the wild-type probe.

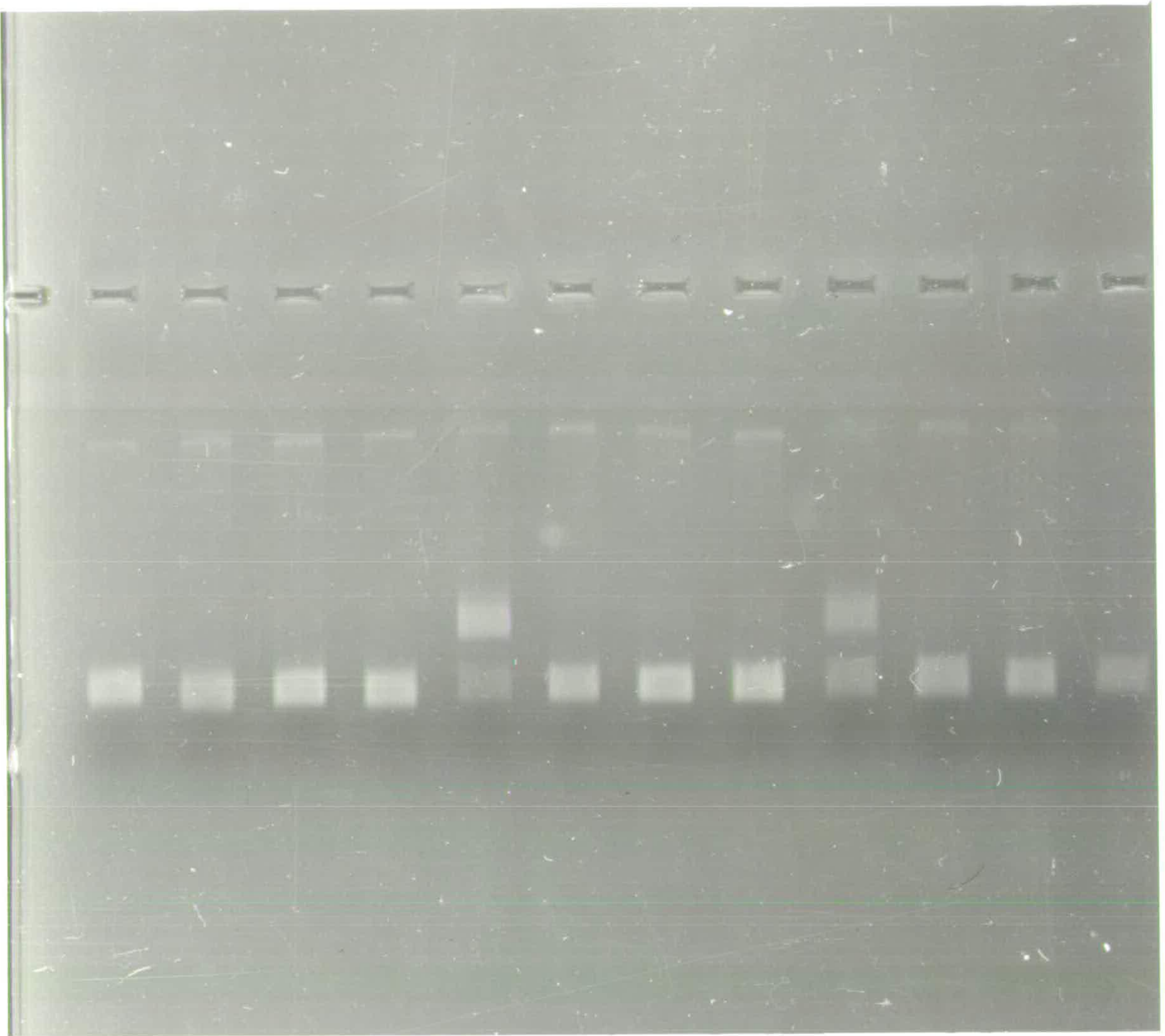


FIGURE 3.6. Selection of single-stranded M13/$\underline{am}^-$ clone required for sequencing by annealing with appropriate single-stranded M13/$\underline{am}^+$ clone. Two of the twelve M13/$\underline{am}^-$ clones clearly annealed with the M13/$\underline{am}^+$ 'probe' and were used for sequencing the am 15 mutant.

(f) Sequencing of the mutant am 15 and two of its revertants, R12 and R15.

After cloning the appropriate Xho I fragments from the three am mutants into the sequencing vector M13 mp8, the Sanger chain termination method (1980) was used to determine the sequence alterations of the three mutants.

Figure 3.7 shows a sequencing gel representing part of the nucleotide sequence of the wild-type am gene and the mutant am 15. The arrow indicates the position in the gel of a band corresponding to a cytosine base in the wild-type sequence which is missing in the am 15 sequence. Apart from this alteration the nucleotide sequences appear to be identical to one another. Thus, as was expected from its reversion with ICR-170, am 15 is a frameshift mutant. The frameshift is a deletion of a cytosine base. From a comparison with the complete nucleotide sequence of the am gene (Kinnaird and Fincham, 1983) it can be seen that the deletion is in codon 56 (AAC) which codes for asparagine.

Figure 3.8 shows a sequencing gel representing the nucleotide sequences of the wild-type am gene and the am 15 R15 revertant. The arrow in the wild-type sequence indicates the cytosine band which is missing in the am 15 mutant. This band is also missing in the R15 revertant. The arrow within the am 15 R15 sequence shows the position of an extra cytosine band which is absent in the wild-type sequence. Thus in the R15 revertant the absence of a cytosine base from codon 56 is compensated for, i.e. the reading frame is restored, by the insertion of an extra cytosine base nine bases downstream.

The nucleotide sequence of the am 15 R12 revertant was determined from the sequencing gel shown in Figure 3.9. Here the arrows indicate the site in the wild-type sequence where a cytosine base is deleted in the am 15 mutant and the site in the revertant sequence where this missing cytosine has been replaced by a guanine leading to the restoration of the correct reading frame.

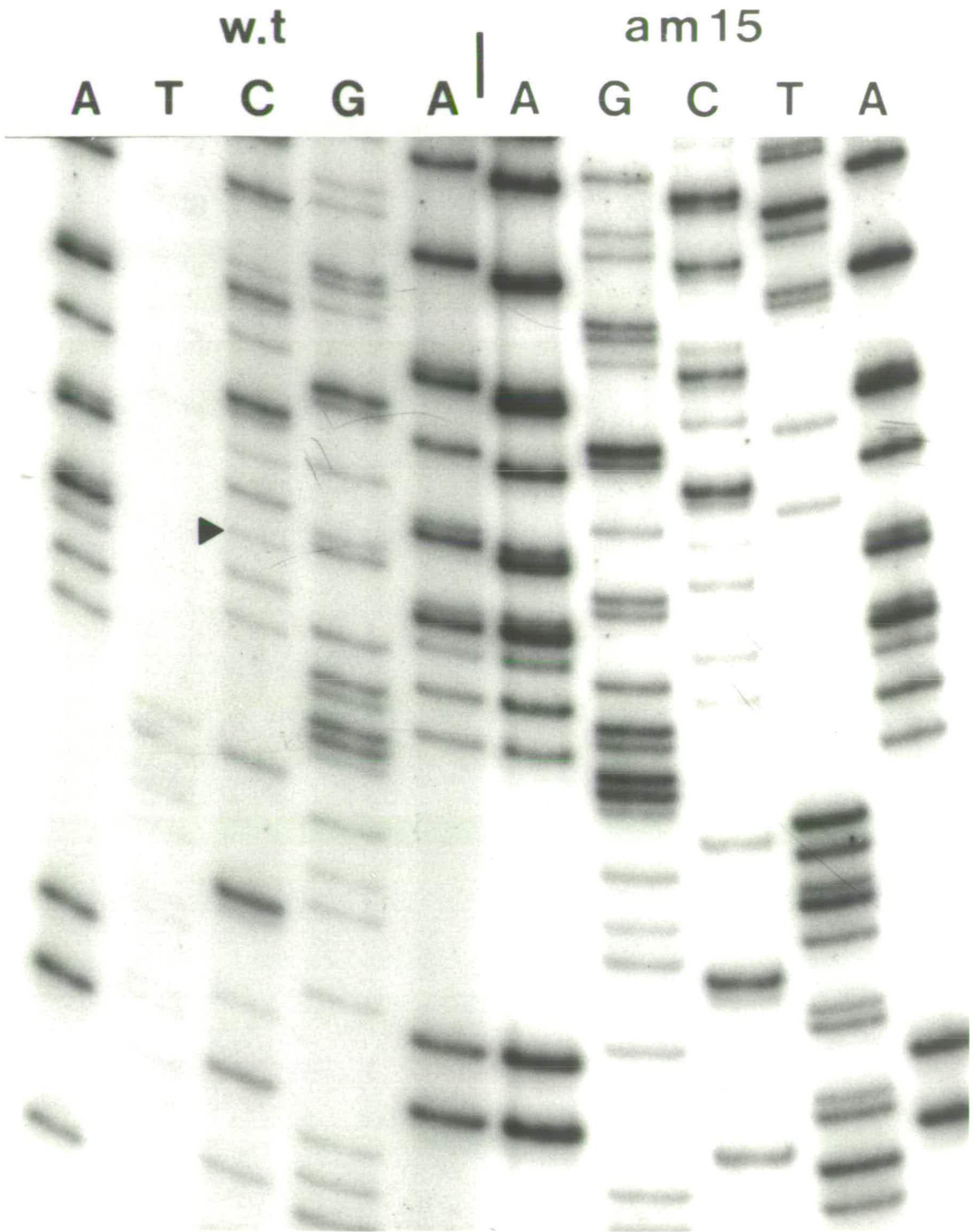


FIGURE 3.7. Sequencing gel representing part of the nucleotide sequence of the am gene from wild-type and am 15. The arrow indicates a band in the C track of the wild-type sequence which is missing from the am 15 sequence.

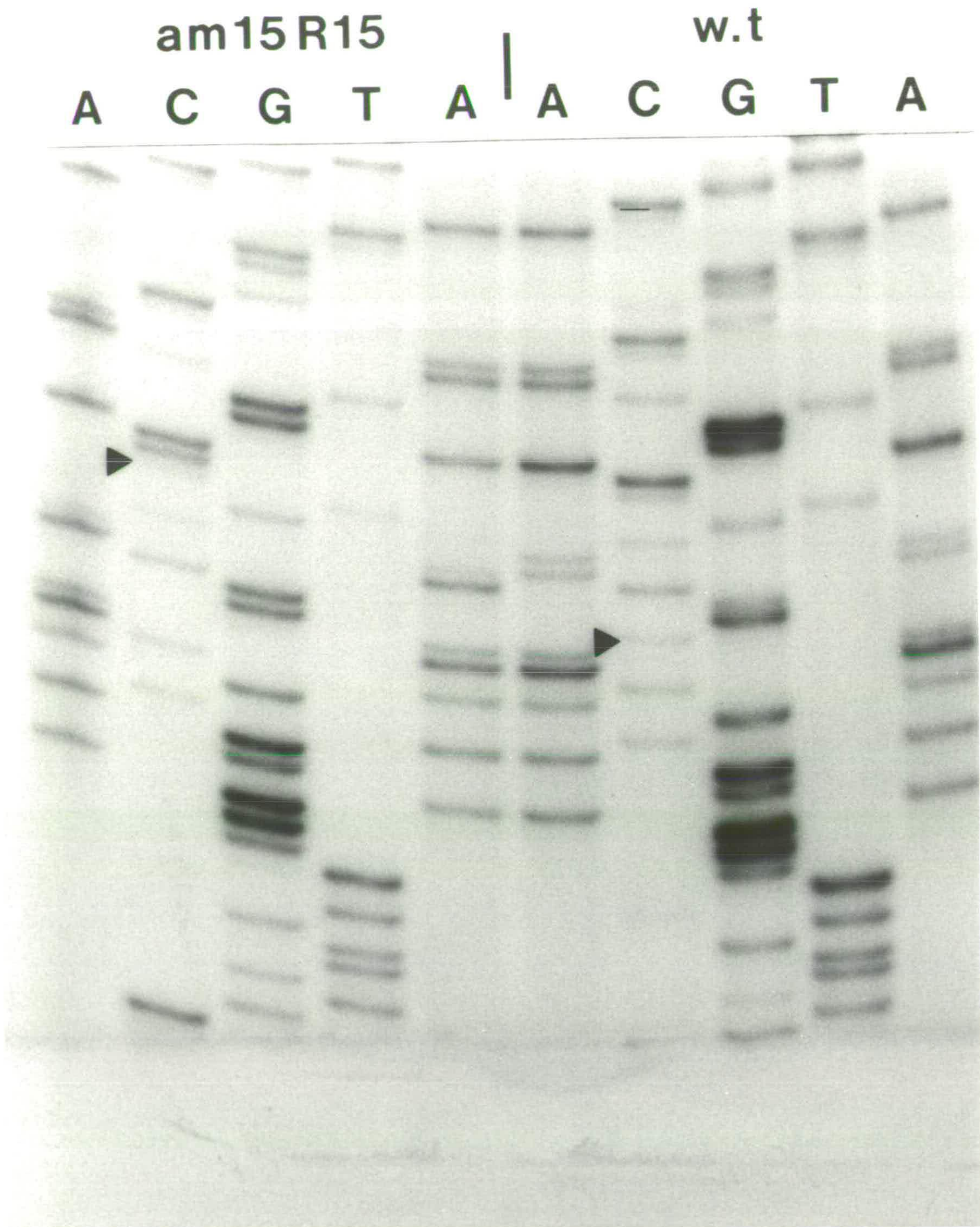


FIGURE 3.8. Sequencing gel of wild-type and am 15 R15. The arrow in the wild-type sequence indicates the C which is missing from am 15 and am 15 R15. The arrow in the am 15 R15 sequence indicates an extra G which is not present in the wild-type sequence.

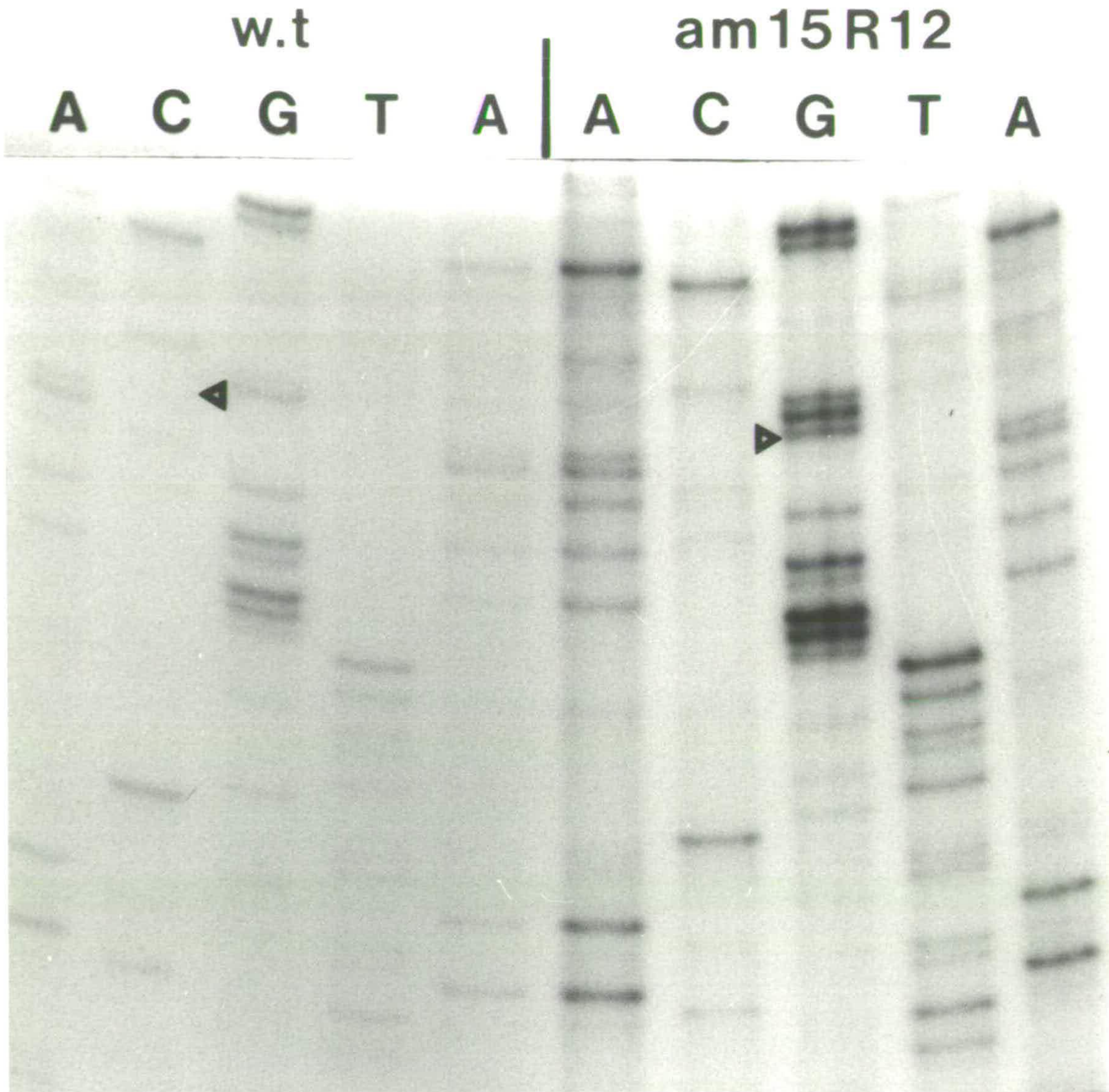


FIGURE 3.9. Sequencing gel of wild-type and am 15 R12. The arrow in the wild-type sequence indicates the C which is missing from am 15 and am 15 R12. The arrow in the am 15 R12 sequence indicates an extra G which is not present in the wild-type sequence.

v) DISCUSSION

ICR-170 Specificity

ICR compounds are known to be potent frameshift mutagens in a range of organisms including Salmonella (Isono and Yourno, 1974), E. coli (Calos and Miller, 1981) and yeast (Donahue et al., 1981). In Neurospora ICR-170 was found to induce predominantly single-site, non-transitional alterations which were thought to be of the frameshift type (Brusick, 1969).

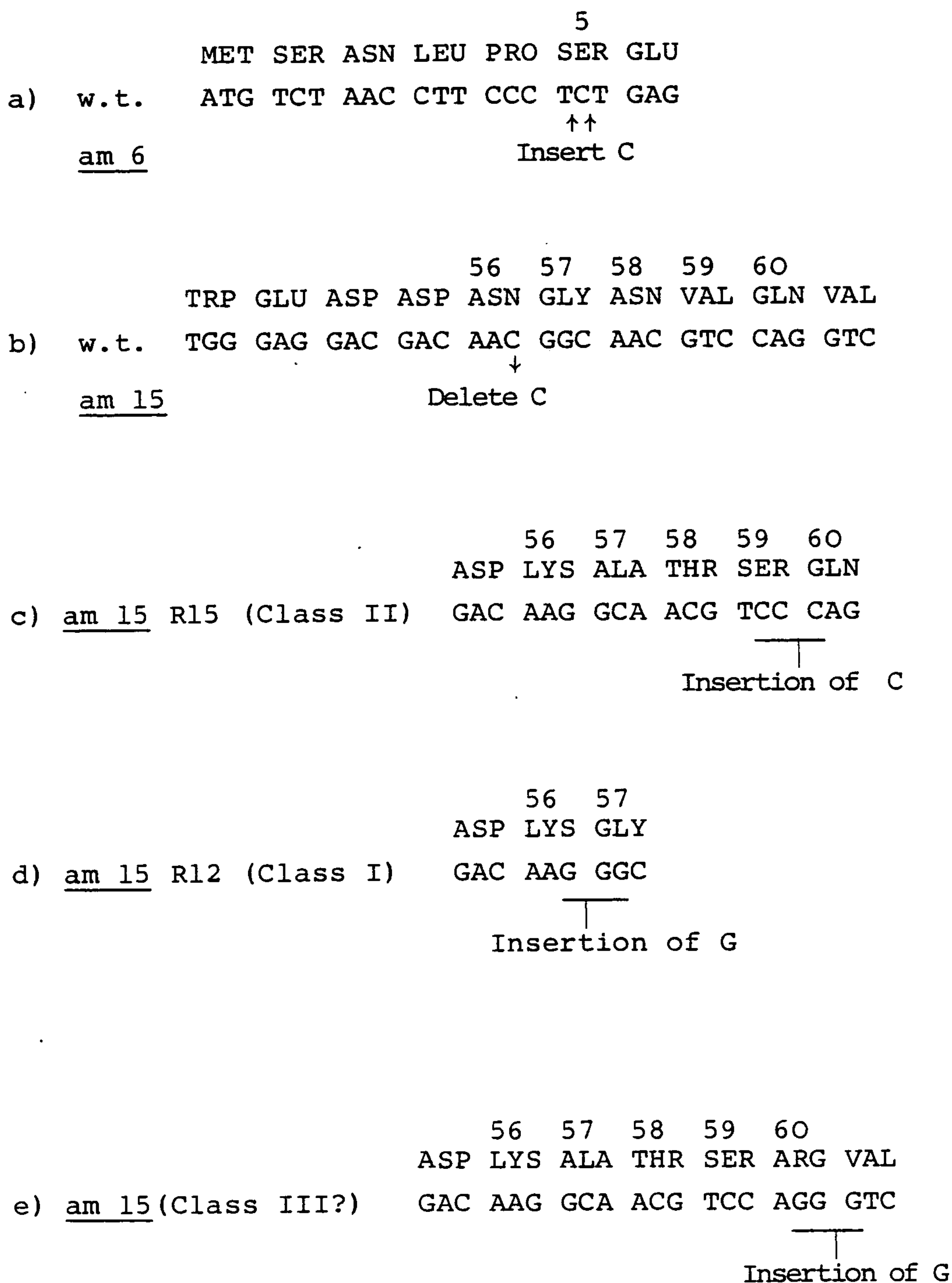
Using the Neurospora am system it was possible to screen directly the ability of ICR-170 to induce frameshift mutations. The am 6 mutant had been shown from amino-acid sequencing studies to be a frameshift mutant with an insertion of a cytosine in the fifth codon of the coding sequence (Fig. 3.10a)(Siddig et al., 1980). However in this study ICR-170 was found to be unable to induce revertants of am 6. In another study (Kinsey and Hung, 1981), ICR-170 was only able to induce revertants of am 6 at a low frequency. Thus ICR-170 is not a potent frameshift mutagen with respect to the am 6 frameshift mutation.

Another am mutant, am 15, had previously been characterised as a frameshift mutant on the basis of its high reversion frequency with ICR-170 and its non-complementation with other am mutants (Fincham, personal communication). In this study ICR-170 was also found to revert am 15 at a high frequency in agreement with the earlier study. However in order to unambiguously characterise am 15 as a frameshift mutant it was necessary to clone and determine the DNA sequence of the mutant.

The sequencing studies revealed that the am 15 mutant represents a deletion of a cytosine from codon 56 (AAC) of the coding sequence (see Fig. 3.10b).

Having shown am 15 to be a frameshift mutant it was of interest to know why ICR-170 should be able to revert am 15

FIGURE 3.10. Sequence alterations of the mutants am 6, am 15, am 15 R15 and am 15 R12



at high frequencies but only be able to revert am 6 at low, and in this study undetectable, levels. From a comparison of the neighbouring sequences of the two mutants (Fig. 3.10) it is not clear why any difference should exist in the revertibility of the two strains with ICR-170. Bearing in mind that ICR compounds show a specificity for inducing frameshifts in runs of G·C base pairs (Calos and Miller, 1983; Donahue et al., 1981) it would seem that both mutants have the potential to revert with ICR-170. In the am 6 mutant there is a run of three cytosines beginning two base-pairs upstream and a pair of cytosines beginning seven base-pairs upstream from the insertion. The run of three cytosines has previously been shown to be a site of UV-induced reversion of the mutant am 6 (Siddig et al., 1980). The downstream sequences of the am 6 alteration are not shown because the presence of an opal nonsense codon sequence (TGA) immediately to the right of the am 6 insertion prevents frameshift reversion downstream.

On the basis of its higher reversion frequency with ICR-170 it might be expected that the am 15 mutation would have far larger numbers of potential ICR-170 mutational sites in its vicinity than the am 6 alteration. However there is only one run of three guanines within forty base-pairs either side of the am 15 mutation and this is eleven base-pairs upstream. In addition there are four pairs of cytosines or guanines within nine base-pairs upstream and thirteen base-pairs downstream. Although this represents a greater potential for ICR-170 mutagenesis in the vicinity of the am 15 mutation it would not seem to be sufficient to account for the difference in reversion frequency between the two mutants, especially when some of the potential ICR-170-induced reversions may involve changes in amino-acid sequence which are incompatible with GDH activity.

Having characterised the am 15 mutant the next step in the investigation of ICR-170 mutagenesis in Neurospora

was to characterise the GDH enzymes of the ICR-170-induced am 15 revertants, and to sequence some of these revertants in order to identify the sequence alterations induced by ICR-170.

Assays of GDH activity and analysis of GDH on non-denaturing poly-acrylamide gels showed that sixteen ICR-170-induced am 15 revertants fell into three distinct classes. Members of class I showed between 62% and 83% of wild-type specific activity in assay system C, showed roughly wild-type stability to heat inactivation, and had about 93% of wild-type electrophoretic mobility on a poly-acrylamide gel.

Class II revertants were similar to class I except for their GDH being completely inactivated after five minutes at 60°C and, probably as a consequence of relative lability having less strongly staining GDH bands on the poly-acrylamide gels.

Class III revertants had the same specific GDH activity as the other revertants, resembled class II in the extreme instability of their GDH at 60°C, but had the slowest GDH electrophoretic mobility, of about 82% of wild-type GDH, on the poly-acrylamide gels.

The observation that all of the revertants had altered electrophoretic mobilities was in agreement with previous studies on ICR-induced am 15 revertants (Fincham, personal communication), and also suggested that ICR-170 was not able to induce wild-type revertants of am 15 in this study.

Time allowed the cloning and sequencing of representatives of only two of the three classes of revertants; R15 from class II and R12 from class I.

The reversion event in R15 was found to be the insertion of a cytosine into the pair of cytosines in codons 59 and 60 (see Fig. 3.10c). This insertion restores the reading frame of the coding sequence but results in the alteration of four amino-acid codons between residues 56 and 59 inclusively. One of these alterations involves the

replacement of asparagine at position 56 with lysine. Since lysine is a positively charged basic amino-acid it is likely that this alteration accounts for the 7% reduction in electrophoretic mobility of the GDH from members of revertant class II. (The samples in the gels are run from negative to positive).

The second revertant to be sequenced was am 15 R12 from revertant class I. This revertant was found to have an insertion of a guanine into the pair of guanine residues in codon 57 (GGC)(see Fig. 3.10d). The overall effect of this reversion event is to replace the cytosine in codon 56 (AAC) of the wild-type sequence with a guanine. Thus the only change in the amino-acid sequence is from asparagine to lysine at position 56. This change must therefore be responsible for the lower specific activity and the slower electrophoretic mobility of class I revertants compared to wild-type. However the change from asparagine to lysine has little noticeable effect on the thermostability of the class I revertants under the conditions employed. This suggests that the unstable nature of the class II revertants in the thermostability assays is due in some way to the change in amino-acid sequence from glycine-asparagine-valine (57-59) in the wild-type GDH to alanine-threonine-serine in the am 15 R15 GDH (see Fig. 3.10c).

The most important observation from these sequencing studies with respect to ICR-170 mutagenesis is that the ICR-induced insertions both occurred in $\frac{-G-G-}{-C-C-}$ sequences. This is the first direct indication that ICR-170 shows the same specificity for inducing frameshift mutations in runs of G·C base-pairs in Neurospora as it does in other organisms. These observations also fit in with Streisinger's model of frameshift mutagenesis involving base slippage and misalignment within repeated sequences during DNA repair or replication (1966). Figure 3.11 shows how the am 15 R12 reversion may have arisen according to Streisinger's model.

FIGURE 3.11. Reversion of am 15 to am 15 R12 as predicted by the Streisinger model (1966).

```
am 15                       -AAGGCA-
                            -TTCCGT-
                               ↓  DNA replication or repair
Direction of synthesis ←GGCA-
                         -TTCCGT-
                               ↓  Base slippage stabilised by ICR-170
                              G
                              GCA-
                         -TTCCGT-
                               ↓  Fixation of extra G by synthesis
                              G
                         -AAGGCA-
                         -TTCCGT-
                               ↓  Insertion of G·C base-pair fixed
                                  at following round of replication
                        -AAGGGCA-
                        -TTCCCGT-
```

Of the sixteen ICR-170-induced revertants of am 15 fourteen fall into the classes represented by am 15 R15 and am 15 R12. Thus it would appear that fourteen out of sixteen ICR-induced mutations at the am 15 site occurred in $\frac{-G-G-}{-C-C-}$ sequences. With this specificity in mind it is possible to speculate on what the alteration in the remaining two revertants might be. One possibility is presented in Figure 3.10e and represents the insertion of a G·C base pair into the next $\frac{-G-G-}{-C-C-}$ sequence downstream from the site of the am 15 R15 reversion. The consequence of such an alteration in terms of amino-acid changes would be the same as that for the am 15 R15 reversion with the additional change of residue 60 from glutamine to arginine. This gain of a basic, positively charged, amino-acid might be sufficient to account for the slower electrophoretic mobility of the class III revertant GDHs with respect to the class II (and class I) revertant GDHs. It is more difficult to speculate about alterations upstream from the am 15 mutation as the sequencing studies do not provide any information on the possible consequences of amino-acid changes on this side of the mutation.

In the light of the evidence that ICR-170 is able to induce frameshifts in the sequence $\frac{-G-G-}{-C-C-}$ it is hard to understand why it is unable to do so in the vicinity of the am 6 mutation, as UV light is able to do, and hence induce am 6 reversions. The reason for this inability may have something to do with the most obvious difference between the am 6 and am 15 mutations i.e. am 6 has a single-base-pair insertion while am 15 has a single-base-pair deletion. It may be that in *Neurospora* ICR-170 is more efficient at inducing insertions than deletions. This could happen if ICR-170 somehow displayed a preference for stabilising looped-out bases in newly synthesised DNA strands rather than in template strands. The template strand may be stabilised in extended conformation by associating

with proteins and hence be much less prone to looping-out than the nascent strand which can be pictured as being unconstrained by protein.

As one might expect if it were an effect of chromatin structure there is no evidence from prokaryotes to suggest a general tendency for ICR compounds to induce insertions rather than deletions, although some studies suggest that there is site-specific preference for either insertions or deletions (Calos and Miller, 1981). However in yeast, as with the am 6 mutant, ICR-170 was not found to induce reversions of a single base insertion mutant, despite the proximity of the sequence GGCCGG, and despite the ability of nitrogen mustard and nitrous acid to induce single base deletions in this sequence (Sherman and Stewart, 1973). Also in yeast ICR-170 was not able to revert a his-4 frameshift mutant, induced by ICR-170, which had an insertion of a G·C base-pair into the sequence $\frac{-G-G-}{-C-C-}$ (Donahue et al., 1981).

At present there is a lack of sequence information on the specificity of ICR-170 mutagenesis in eucaryotes. This study has gone some way to remedying this situation but more sequences studies will be required before we can obtain a good understanding of the factors involved in the mutagenic specificity of ICR-170 in eucaryotes.

Frameshift specificity of UV, NQO and EMS

The three mutagens UV, NQO and EMS were screened for their ability to revert the two frameshift mutants am 6 and am 15.

UV was able to revert am 6 with high efficiency up to a frequency of 25 x 10^{-6} surviving conidia, but was only able to revert am 15 at a relatively low frequency of 0.2 x 10^{-6} surviving conidia. This is in agreement with Siddig et al. (1980) who obtained a reversion frequency of 20-25 x 10^{-6} surviving conidia with am 6 and Seale (1968) who

obtained no revertants of am 15 after UV treatment. The reason for the very different responses of these two mutants to UV is not clear. It may be that UV is able to induce deletions more easily than insertions in Neurospora. In E. coli twenty-three UV-induced frameshifts in the lac I gene all turned out to be single-base deletions, and throughout the lac I gene single-base delations were 10-20 fold more frequent than single-base additions after UV treatment (Miller, 1983). It is conceivable that some aspect of repair of UV damage can lead to a bias for deletions over additions, perhaps through the removal of damaged bases and subsequent fixation of this loss. However it is not possible to draw any firm conclusions on UV-induced frameshift specificity in Neurospora on the basis of reversion studies with only two mutants.

In contrast to UV, NQO was able to revert both frameshift mutants at a similar, albeit a rather low, frequency. This suggests that despite the UV-mimetic properties of NQO with respect to inactivation and mutation induction of UV-sensitive strains of Neurospora (Inoue et al., 1980), there are important differences between UV and NQO with respect to the induction of frameshift mutations.

In bacteriophage T4 NQO was unable to revert two frameshift mutants (Ishizawa and Endo, 1970) while in Salmonella NQO was able to delete the doublet CG from the sequence CGCGCGCG (Isono and Yourno, 1974). In yeast NQO was unable to revert a frameshift mutant with the sequence AAAA in the near vicinity (Prakash et al., 1974). It would appear therefore that NQO is not a good frameshift mutagen but is able to induce frameshifts at low frequencies at particular sites. No investigation of the site specificity of NQO frameshift mutagenesis has been carried out. It would be interesting to know whether NQO showed the same specificity for G·C base-pairs in frameshift mutagenesis as it does in base-substitution mutagenesis, or whether the

only specificity is for repetitive DNA sequences.

EMS, an alkylating agent, was able to revert the am 6 mutant at a relatively high frequency but there was little evidence of any reversion of am 15. Thus EMS displays the same reversion as UV with respect to these two mutants. There is little information available on the ability of EMS to induce frameshift mutations in other systems. In yeast EMS was unable to revert an insertion frameshift mutant although other alkylating agents such as nitrogen mustard and MMS were able to do so (Sherman and Stewart, 1973). It is thought that alkylating agents may induce frameshift mutations through the formation of apurinic gaps. The main products of alkylation of DNA are N-7 alkylguanine and N-3 alkyladenine. These modifications labilise the N-glycosylic bands leading to increased rates of spontaneous depurination (Lawley and Brookes, 1963). In addition DNA glycosylases have been found in *E. coli* which specifically catalyse the removal of N-3 and N-7 alkylpurines, leaving apurinic gaps which are repaired by apurinic endonucleases (Lindahl, 1982). However apurinic gaps are found to be mutagenic in cells in which the SOS response has been induced (Schaaper *et al.*, 1982). It is conceivable that apurinic gaps could be fixed during error-prone repair and hence give rise to single-base deletions of purines. Thus alkylating agents might be expected to revert single-base insertions preferentially, and because N-7 alkylguanine is the major product, might be expected to induce predominantly deletions of G·C base-pairs.

The results of this study show that EMS reverts a single-base insertion with far better efficiency than a single-base deletion. It would be interesting to carry out sequencing studies on EMS-induced revertants of am 6 to see if reverting deletions were of G·C base-pairs. It would also be interesting to screen other alkylating agents for their ability to revert am 6 and am 15. Such studies would probably throw some light on the general mechanisms of alkylating agent-induced frameshift mutagenesis in *Neurospora*.

CHAPTER IV: CONCLUSIONS

This piece of work set out with the straightforward aim of investigating the mutagen specificity of five well-studied mutagenic agents in Neurospora crassa using a tester system of six well-characterised mutants of the am gene. Four of the mutants, am 1, am 3, am 7 and am 17, were used to screen the ability of the mutagens UV, NQO, EMS and nitrous acid, to induce various types of base substitution. The other two mutants, am 6 and am 15, were used to screen the ability of the mutagens ICR-170, UV, NQO and EMS to induce frameshift mutations. In the course of the study am 15 and two ICR-induced revertants of am 15 were cloned and sequenced.

The overall conclusion that could be drawn from these studies is that, on the whole, the five mutagens showed the same specificity in Neurospora as they do in yeast and various prokaryotic systems.

UV was shown to be able to induce A·T → G·C transitions and, almost certainly, G·C → A·T transitions in the strains am 1, am 7 and am 3. From an analysis of UV-induced revertants of the nonsense mutant am 17 it was found that UV is able to induce transversions of A·T and G·C base pairs but shows a preference for inducing A·T → G·C transitions at this site. From the studies on the two frameshift mutants it was found that UV is able to revert a single-base insertion mutant but did not induce any revertants of a single-base deletion mutant. Whether this finding is of significance with respect to a mechanism of UV-induced frameshift mutagenesis, or whether it is simply an example of differential UV mutability of two sites within the am gene, is not clear.

NQO was found to be able to induce A·T → G·C and probably G·C → A·T transitions in reversion studies with am 1, am 7 and am 3. However in studies with the am 17 nonsense

mutant it was found that NQO displayed a marked preference for the induction of G·C transversions over transitions and transversions of A·T base-pairs. Out of twenty-seven mutational events twenty-four appeared to involve G·C transversions. At the same time it was concluded that the am 17 nonsense mutant is an amber (TAG) mutant. This conclusion was supported by later evidence from sequencing studies (Kinnaird and Fincham, 1983) which showed that the am 17 nonsense codon was derived from the wild-type codon CAG. This is the first nonsense mutation to be characterised in *Neurospora*. NQO was found in reversion studies with am 6 and am 15 to be a low-level inducer of frameshift mutations.

EMS was found to induce revertants only of the am 3 mutant out of the four mutants used to screen for base-substitutions. The only base-substitution which the mutants am 1, am 7 and am 17 can not screen for is a G·C → A·T transition. However the mutant am 3 reverts to wild-type via a G·C → A·T transition and a proportion of the EMS-induced am 3 revertants were apparently wild-type. Thus EMS could only be shown to be able to induce G·C → A·T transitions in this system. In studies with the frameshift mutants EMS appeared to induce deletions with greater ease than insertions. This may be the consequence of fixation of apurinic gaps induced by EMS in the *Neurospora* DNA.

Nitrous acid was a poor mutagen in this system. The only base-substitutions which this mutagen was able to induce in reversion studies with the four mutants was A·T → G·C transitions. No reversions due to G·C transitions or any type of transversion were observed. Nitrous acid is thought to induce transitions of A·T base-pairs through its ability to deaminate adenine to hypoxanthine which then pairs with cytosine. Nitrous acid was not tested on the frameshift mutants.

ICR-170 is known to be a potent frameshift mutagen. The primary aim of the studies with the mutants *am 6* and *am 15* was to investigate the site-specificity of ICR-170-induced frameshift mutagenesis in *Neurospora*. Fourteen out of sixteen ICR-170-induced revertants of *am 15* were characterised as being insertions of a G·C base-pair into one of two -G-G-/-C-C- sequences. No revertants of *am 6* were obtained after ICR-170 treatment. *am 6* had previously been shown to revert via a deletion of a G·C base-pair from the sequence -G-G-G-/-C-C-C- after UV treatment (Siddig *et al*., 1980). It is not clear why ICR-170 was unable to induce this alteration in these studies, especially as a closely related compound, ICR-191, is able to induce the same alteration in *E. coli* (Calos and Miller, 1981). Repetitive DNA sequences tend to be hotspots for frameshift mutagenesis as predicted by the Streisinger model (1966). The ability of ICR compounds to induce frameshifts specifically in runs of G·C base-pairs is thought to be due to the preferential reaction of the alkylating side groups of ICR compounds with the N-7 position of guanine.

While studies of this sort can provide a great deal of useful information on the mutagenic specificity of mutagens, they do have one serious drawback. When reversion of well-characterised mutants is used to screen the specificity of mutagens the study is inevitably restricted to a few well-defined locations in the DNA. As can be seen from the results of reversion studies with the mutants *am 1* and *am 7* mutation rates can differ quite markedly over short distances for the same base-pair alteration. Thus it is unsafe to draw firm conclusions from information gleaned from a limited number of sites.

The most satisfactory way of studying mutagen specificity is to induce forward mutations to loss of function in the gene being used for study, and then to sequence these mutations. Using this method there are fewer restrictions

on the types or locations of induced alterations. It is only recently however that the technology required for this type of study has become available. With the advent of rapid cloning and sequencing techniques it should be possible to characterise large number of mutants induced by various mutagens.

Using the forward selection procedure of Kinsey (1977) it is possible to induce forward mutations in the am gene and to clone and sequence these mutants using the protocol outlined in the Methods section of Chapter III. This system is at present being used to investigate more fully the mutagenic specificity of ICR-170 in Neurospora crassa, and should be available for studies of other mutagens in the future.

ACKNOWLEDGEMENTS

In the cloning and sequencing studies Dr J. Kinnaird kindly provided λL47 and M13mp 8 DNA and any required am^{+}/λ and am^{+}/M13 clones. M. Keighren purified the *am*15I12/λ clone from the positive signal on the clone selection plates. A. Campbell provided occasional technical assistance.

I am grateful for all the helpful advice from the members of the mutagenesis group and Professor Fincham's group, and am especially indebted to Dr J. Kinnaird for patiently teaching me all of the cloning and sequencing techniques.

I should like to thank my two supervisors, Professor J.R.S. Fincham and Dr B.J. Kilbey, for their excellent supervision and reading of the manuscript.

Lastly I should like to thank my squash partner for helping to keep me healthy in body and especially my wife Julie for keeping me healthy in mind.

Throughout my Ph.D. I was in receipt of an SERC grant.

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